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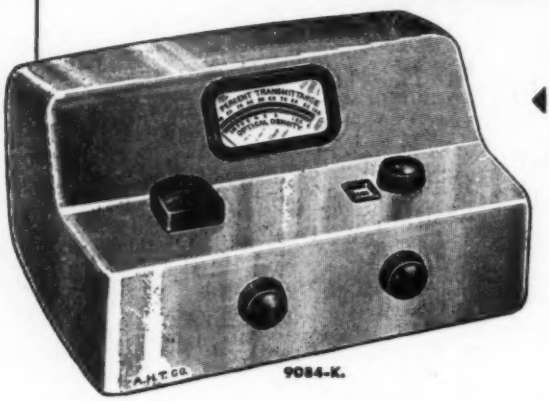

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THE SENSITIVITY OF DEVELOPING HABROBRACON TO OXYGEN [1]

A. M. CLARK AND E. B. HERR, JR.[2]

Marine Biological Laboratory, Woods Hole, Mass., Brookhaven National Laboratory, Upton, N. Y., and University of Delaware, Newark, Delaware

Injury from high tensions of oxygen has been reported for a wide range of plants and animals. These have included chromosome breakage (Conger and Fairchild, 1952) and numerous non-nuclear changes (Stadie, Riggs and Haugaard, 1944). Among insects, toxicity from oxygen, as manifested by a decrease in wing beat frequency and reduced viability, has been shown for adults of *Drosophila azteca* exposed to up to ten atmospheres of oxygen (Williams and Beecher, 1944). *Drosophila melanogaster* exposed as embryos exhibited a slight lag in development, but without concomitant effects on viability (Glass and Plaine, 1952). During investigations on the parasitic wasp, *Habrobracon*, it was found that pupal development was arrested by high tensions of oxygen. Since reports of such effects are meager, it was decided to investigate this phenomenon in more detail.

Materials and Methods

The methods of rearing *Habrobracon* and of experimentation have appeared in previous publications (Clark and Mitchell, 1951). Females from stock No. 33 were crossed to males from stock No. 1. Diploid daughters were obtained from the fertilized eggs and haploid sons from unfertilized eggs. The male and female offspring were grown together in the same culture and treated at the same stage of development. All cultures were raised at 30° C.

The stages of development at which *Habrobracon* were treated and the chronological ages that coincide with these stages are shown in Figure 1. "Larva-in-cocoon" (3–4½ days) is the period from the enclosure of the larva in the cocoon to the appearance of the meconium; "prepupa" (4½–5½ days) is the stage from the egestion of the meconium to the presence of external appendages.

The wasp cultures were exposed to air, oxygen, nitrogen, and carbon dioxide in chambers about 100 cm.^{3} in volume. The gases were obtained from commercial cylinders. No attempt was made to remove impurities since previous investigators such as Conger and Fairchild (1952) found no objectionable effects from trace contaminants in commercial cylinders.

[1] Research carried out (in part) under A.E.C. Contract AT(30-1)-1409 with the Marine Biological Laboratory and at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[2] Present address: Department of Biochemistry, Cornell University, Ithaca, N. Y.

The deleterious effects were measured by determining the incidence of adults emerging from cocoons (the eclosion ratio), the occurrence of structural abnormalities among these adults and the arrest of development.

Results

Groups of individuals that ranged from the larva-in-cocoon stage to the pigmented pupal stage were exposed to one atmosphere of oxygen or of nitrogen for one hour at 30° C. Groups exposed to oxygen as larvae in cocoons or as young prepupae showed no injurious effects. Groups treated as older prepupae or as pupae were injured, white pupae (6–6½ days) being the most sensitive (Table I,

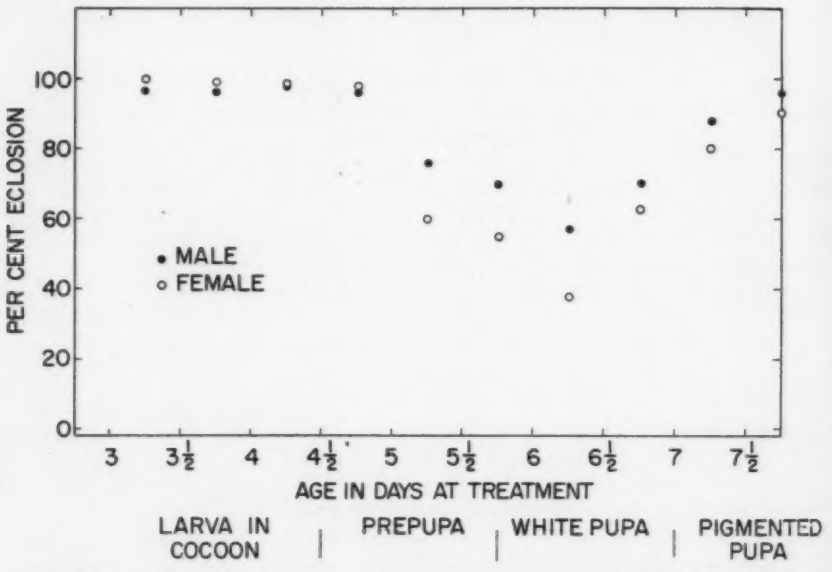


Figure 1. The relation of stage of development to oxygen sensitivity in Habrobracon. The deleterious effect is expressed as per cent eclosion following exposure to oxygen (100 per cent) for one hour. Data from Table I.

Fig. 1). The exposure to oxygen decreased the incidence of individuals developing to the adult stage and emerging from cocoons. Arrest of development usually occurred during the pigmented pupal stage, although such pupae remained alive for at least two weeks after eclosion of controls. An appreciable number of the adults which did emerge show wing and antennal abnormalities. The eclosion per cent for females was significantly less than for comparable males. None of the deleterious effects described for oxygen were found following exposure to nitrogen at any stage of development. The eclosion ratios for nitrogen-treated groups were not significantly different from air controls.

The preceding experiment shows that the oxygen sensitivity of *Habrobracon* varies markedly during development. Next, groups of individuals in a sensitive

TABLE I

Eclosion ratios for males and females after exposure to oxygen or nitrogen at different stages of development

Age (days) at treatment	Oxygen				Nitrogen			
	Males		Females		Males		Females	
	eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted
3–3½	.97	29	1.00	11				
3½–4	.96	182	.99	142	.90	50	.92	39
4–4½	.98	186	.98	173	.98	43	.96	49
4½–5	.96	185	.98	168	1.00	36	.92	57
5–5½	.76	286	.60	270	.96	50	1.00	33
5½–6	.70	354	.55	404	.98	58	.97	36
6–6½	.57	402	.38	452	.99	65	.98	41
6½–7	.70	371	.63	361	.93	44	.94	33
7–7½	.88	56	.80	39	.96	49	.97	35
7½–8	.96	50	.90	33	.92	53	.95	42
air controls	.96	439	.97	340				

period (white pupal stage) and during a resistant period (larva-in-cocoon stage) were exposed to air, oxygen, nitrogen or carbon dioxide at one or at two atmospheres pressure. The groups were exposed at one atmosphere for 15 minutes or at two atmospheres for 5 minutes.

Eclosion ratios following treatment of the larva-in-cocoon stage with air, oxygen, nitrogen or carbon dioxide at one or at two atmospheres indicate no deleterious effects on development or eclosion (Table II). Following exposure of white pupae to one atmosphere of nitrogen, eclosion is not significantly lower than air controls, but after one atmosphere of oxygen or of carbon dioxide eclosion is significantly lower (Table II) and after two atmospheres of oxygen or carbon dioxide there is a still greater decrease in the incidence of adults eclosing. A decrease in per cent eclosion occurs following treatment in two atmospheres of air over one atmosphere of air. Treatment in two atmospheres of nitrogen shows a non-significant decrease in eclosion for males and a small but significant decrease for females. Repetition of these experiments showed no decrease in per cent eclosion following treatment with nitrogen at two atmospheres versus one atmosphere, but did show marked differences between one and two atmospheres for air, oxygen and carbon dioxide. The difference shown for nitrogen between female pupae treated at one and at two atmospheres (Table II) is therefore spurious. Since treatment in two atmospheres of nitrogen shows no deleterious effects, it would seem that pressure itself is not responsible for the effects. The eclosion ratios following treatment in one atmosphere of oxygen or carbon dioxide are less for females than for males.

The development of white pupae exposed to two atmospheres of oxygen was arrested immediately. They remained as white pupae for one week, then started to develop pigmentation. These pupae remain alive in this arrested condition for at least three weeks after controls have emerged as adults. Observations of the arrested pupae were not continued after this period. Of comparable carbon

TABLE II

Eclosion ratios for males and females after exposure to air, oxygen, nitrogen or carbon dioxide

Gas	Sex	Larva-in-cocoon stage				White pupa stage			
		1 At.		2 At.		1 At.		2 At.	
		eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted
Air	♂	.91	54	.77	44	.90	93	.64	55
	♀	.92	64	.82	50	.90	112	.68	111
Oxygen	♂	.92	52	.85	54	.55	145	.03	77
	♀	.92	75	.86	56	.35	124	.01	74
Nitrogen	♂	.87	76	.91	45	.92	102	.82	60
	♀	.87	103	.90	40	.93	99	.75	87
Carbon dioxide	♂	.85	106	.83	40	.67	161	.23	64
	♀	.91	155	.95	59	.46	213	.09	57

dioxide-treated pupae, some continued to develop to the pigmented pupal stage before becoming arrested while others lagged for a few days as white pupae. Oxygen-treated pupae appear to be affected more than comparable pupae exposed to carbon dioxide.

Following exposure for five minutes to two atmospheres of nitrogen or to two atmospheres of oxygen, 25 white pupae were placed in each Warburg flask and oxygen uptake measured. Oxygen uptake was recorded in terms of the displacement of the Brodie's fluid, which serves sufficiently well for comparison of the effects of the gases. Upon repetitions of this experiment flasks used to measure oxygen-treated pupae were used to measure the oxygen consumption of nitrogen-treated pupae. Since the same results were obtained in all cases, errors due to slight volume differences in flasks or in the bore of the manometer are irrelevant. Neither two atmospheres of nitrogen nor one atmosphere of oxygen affected oxygen uptake. Two atmospheres of oxygen, however, resulted in an immediate and marked decrease in oxygen uptake (Fig. 2). Exposure to two atmospheres of carbon dioxide produced only a slight decrease in oxygen uptake. No decrease in oxygen uptake was observed following exposure of larvae-in-cocoons to oxygen or carbon dioxide at normal or increased pressure. The foregoing experiment shows a marked and immediate decrease in the oxygen uptake of white pupae exposed to two atmospheres of oxygen. Further work of a more quantitative nature has been planned.

DISCUSSION

Most studies have shown that the metabolic rate of immature insects is lowered by exposure to low tensions of oxygen (Wigglesworth, 1950). Effects of high oxygen tension on development have been reported by Glass and Plaine (1952) who found a slight lag in eclosion from cocoons without concomitant effects on viability for *Drosophila* exposed as embryos. The present work shows that a

decrease in metabolism can be obtained by exposure to high tensions of oxygen. Pupae of *Habrobracon* are arrested in their development following such treatment and oxygen consumption is reduced. The development of pupae of the *Cecropia* silkworm is slightly delayed following exposure to 100 per cent oxygen for 21 days and slightly abnormal adults are produced (Schneiderman and Williams,

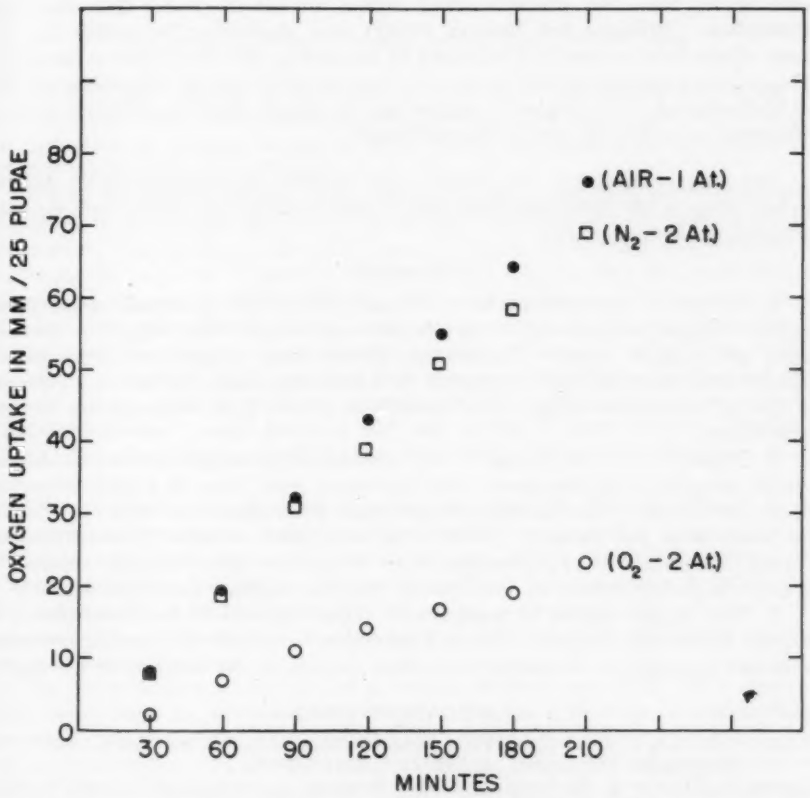


FIGURE 2. The oxygen consumption of white pupae following exposure for five minutes to two atmospheres of oxygen and of carbon dioxide. Ordinate: millimeters displacement of fluid in the arm of the manometer. Twenty-five pupae were used in each flask.

1954). In neither *Drosophila* nor *Cecropia* has treatment with oxygen at higher pressures been reported.

Work on animals as unrelated as rats, turtles and *Drosophila* adults has shown that younger animals are more resistant to injury by oxygen than older animals (Williams and Beecher, 1944). In *Habrobracon* this sensitivity is stage-dependent, since white pupae are more sensitive than the younger larvae or the older pig-

mented pupae. The sensitivity may be also sex-dependent since males are more resistant than females.

The oxygen-sensitive, white pupal, stage is also injured by exposure to carbon dioxide and the oxygen-resistant, larva-in-cocoon, stage is unaffected by carbon dioxide. The injury from carbon dioxide is probably not due to anoxia because the duration of exposure was short. An effect from carbon dioxide is indicated since injury from two atmospheres of carbon dioxide is greater than from one atmosphere. Williams and Beecher (1944) have shown for *Drosophila* that the toxic effects from oxygen are increased as the carbon dioxide tension is increased. The present observations can do no more than to point out the extreme sensitivity of *Habrobracon* white pupae to oxygen and to carbon dioxide. Further work is necessary to explain the mechanisms involved.

The authors thank Dr. John Buck of the National Institutes of Health and Dr. Robert King of the Brookhaven National Laboratory for their careful and vigorous criticism of this manuscript.

SUMMARY

1. Groups of *Habrobracon* were exposed during post-embryonic development to air, nitrogen, oxygen and carbon dioxide and studied with regard to development and oxygen uptake. Deleterious effects from oxygen and from carbon dioxide were observed after exposure at the white pupal stage, but not after exposure at the larva-in-cocoon stage. No deleterious effects from exposure to nitrogen were found.

2. Exposure of white pupae to one atmosphere of oxygen or carbon dioxide caused cessation of development at the pigmented pupal stage in a high proportion of the individuals. The incidence of emergence from cocoons as adults was higher for males than for females. Adults with wing and antennal abnormalities occurred. Exposure to two atmospheres of oxygen or carbon dioxide resulted in greater deleterious effects on development than did exposure to one atmosphere.

3. The oxygen uptake of unpigmented pupae exposed to two atmospheres of oxygen is markedly reduced. No such reduction was observed following treatment with two atmospheres of nitrogen or carbon dioxide or one atmosphere of oxygen.

LITERATURE CITED

CLARK, A. M., AND C. J. MITCHELL, 1951. Radiosensitivity of haploid and diploid *Habrobracon* during pupal development. *J. Exp. Zool.*, **117**: 489–498.

CONGER, A. D., AND L. M. FAIRCHILD, 1952. Breakage of chromosomes by oxygen. *Proc. Nat. Acad. Sci.*, **38**: 289–299.

GLASS, B., AND H. L. PLAINE, 1952. The role of oxygen concentration in determining the effectiveness of X-rays on the action of a specific gene in *Drosophila melanogaster*. *Proc. Nat. Acad. Sci.*, **38**: 697–705.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1954. The physiology of insect diapause. IX. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull.*, **106**: 238–252.

STADIE, W. C., B. C. RIGGS AND N. HAUGAARD, 1944. Oxygen poisoning. *Amer. J. Med. Sci.*, **207**: 84–114.

WIGGLESWORTH, V. B., 1950. The principles of insect physiology. Methuen and Co., Ltd., London, pages 414–417.

WILLIAMS, C. M., AND H. K. BEECHER, 1944. Sensitivity of *Drosophila* to poisoning by oxygen. *Amer. J. Physiol.*, **140**: 566–573.

STUDIES ON THE ACROSOME.
III. EFFECT OF CALCIUM DEFICIENCY[1]

JEAN C. DAN

Misaki Marine Biological Station, Misaki, Kanagawa-ken, Japan

The idea that calcium is necessary for fertilization was clearly formulated by Jacques Loeb (1914, 1915), who suggested, as a possible explanation, that it might be an ingredient of a sort of cement which holds the sperm to the egg surface in the first stage of sperm attachment.

A very different and more complicated role in the fertilization reaction has been attributed to the calcium ion by Heilbrunn, as a corollary of his general theory of stimulation (for summary, see Heilbrunn, 1937, chaps. IX and XXXVII). In particular the work of his student, Moser (1939), showed that the breakdown of the cortical granules of sea urchin eggs, which is the precursor of membrane elevation and subsequent developmental processes in normal fertilization and artificial parthenogenesis, does not occur when the eggs are activated in oxalated or citrated media. Yamamoto (1954), pushing this analysis a step further, has found that in teleost eggs calcium ions are necessary for the propagation of an invisible "fertilization-wave" which precedes and sets off the cortical changes. Sugiyama (1953) has shown that a similar wave of excitation occurs on fertilization in the sea urchin egg.

This demonstrated importance of calcium for the first detectable change in the egg cortex at the time of activation, together with the fact that spermatozoa are normally active and attack the unfertilized egg surface vigorously even in the absence of calcium, has led to a general assumption that the failure of fertilization in Ca-free media is due to the inability of the egg to respond to the stimulus of the spermatozoan, rather than to any reduction of spermatozoan stimulating capacity in the absence of calcium.

No doubt because of the lack of a suitable method, very little effort seems to have been made to determine the effect of calcium deficiency on spermatozoa, although Tyler mentions the fact that "the absence of (calcium) interferes with the agglutination of sea urchin sperm" (1948, p. 210). The reaction of the sea urchin sperm acrosome to egg-water (Dan, 1952), however, in which at least part of the acrosomal substance is released, accompanied by the projection of a fibrous structure, provides one clear-cut criterion for comparing the effects on the individual spermatozoan of various non-fatal experimental conditions, including calcium deficiency.

The observations presented in this paper will show that calcium deficiency prevents the acrosome reaction in response to various stimuli, while it tends to enhance the agglutinating effect of egg-water and prevents the loss of fertilizing capacity after reversal of agglutination.

[1] This work was supported by a grant-in-aid from the Ministry of Education (Minkan Kenkyu Hi).

Materials and Methods

Among the sea urchins easily available at Misaki, the spermatozoa of the three species of regular sea urchins, *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus, Anthocidaris* (*Heliocidaris*) *crassispina* and *Pseudocentrotus depressus,* show typical reversible agglutination when mixed with the egg-water of their respective species; the following observations were made on the gametes of these three species.

Eggs were obtained by the introduction of isotonic KCl into the body cavity. In the case of "Ca-low egg-water," the animal was rinsed with Ca-free artificial sea water and made to shed its eggs directly into this solution. The egg suspension was sometimes warmed to about 30° C. to hasten dissolution of the jelly, or the eggs were centrifuged at 2000 × gravity for 5 minutes and the empty jelly hull suspension warmed to about 50° C. for a few minutes. In every case the egg-water was filtered before use.

Control egg-water solutions were obtained either by centrifuging the jelly from an equivalent volume of eggs in sea water, or by adding sufficient isotonic $CaCl_2$ to the Ca-low egg-water to bring its calcium content to that of normal sea water.

Spermatozoa were collected "dry" as shed following KCl stimulation, or as they exuded from excised testes. Care was always taken to reduce contamination by sea water or body fluids. In most of the experiments, 1 or 2% of dry sperm were suspended in Ca-free solution or filtered sea water. Spermatozoa were always freshly suspended immediately before the addition of egg-water, unless otherwise specified.

Although many biological systems are sensitive to minute changes in calcium concentration, the sperm acrosome was found to be unexpectedly insensitive in this respect. For example, in *Pseudocentrotus,* the acrosome reaction was still suppressed in all sperm at 25% of the normal calcium concentration, only about half the acrosomes reacted at 50% calcium concentration, and response of all the acrosomes was only obtained at 67% of the calcium concentration of normal sea water. In view of this fact, no effort was made in these experiments to remove the last traces of calcium from the experimental solutions by the use of oxalate or citrate, and the term "Ca-free" has therefore been avoided in referring to egg-water solutions and sperm suspensions. However, it should be pointed out that the calcium content of these media was below the threshold required for fertilization to take place in them.

As in the first study of this series, sperm suspensions were fixed by the addition of neutralized formalin. Preparations for electron microscopy were made directly from these suspensions; washing out of the sea water salts was done after mounting on the collodion membranes. Some of the preparations were shadowed with Cr_2O_3; the electron microscope used was a Hitachi Standard, operating on 50 KV.

Results

The reversible agglutination of the spermatozoa of these three sea urchin species follows the usual pattern, which has been described in detail by Jacqus Loeb (1914) for two species of *Strongylocentrotus.* While there are certain details of the agglutination reaction which seem to be peculiar to one or another of the species

under consideration, description of these will be omitted, and the major aspects of the reaction common to all the species will be reported. Unless otherwise specified, the statements will apply to all three species.

Attempts to treat the agglutination reaction quantitatively run into many difficulties because of the lack of a clear end-point in the reaction itself and the virtual impossibility of standardizing the reactants (see Goldforb, 1929a, 1929b, 1929c, for a detailed discussion of individual variation among sea urchin gametes and the effect of ageing of spermatozoa on agglutination). In order to report the phenomenon with any semblance of clarity, therefore, it has seemed necessary to disregard the borderline data and concentrate attention on the cases which consistently give positive or negative results.

Spermatozoa suspended in Ca-free sea water

If spermatozoa freshly suspended in Ca-free sea water are compared under the high power of the phase contrast microscope with a similar sample in ordinary sea water, a striking difference is immediately observable between the behavior of the sperm in the two suspensions. The majority of the spermatozoa in sea water are either trapped against the glass surfaces on their sides or have undergone the acrosome reaction on contact with the glass and are rotating around their tips, held by the acrosome process (Dan, 1952). In the Ca-low preparation, on the other hand, all the spermatozoa are found to be in vigorous locomotion, describing uniformly-sized circles against the glass surfaces. When these have stopped moving sufficiently to permit close observation, it can be seen that none of them has reacted to the contact stimulus.

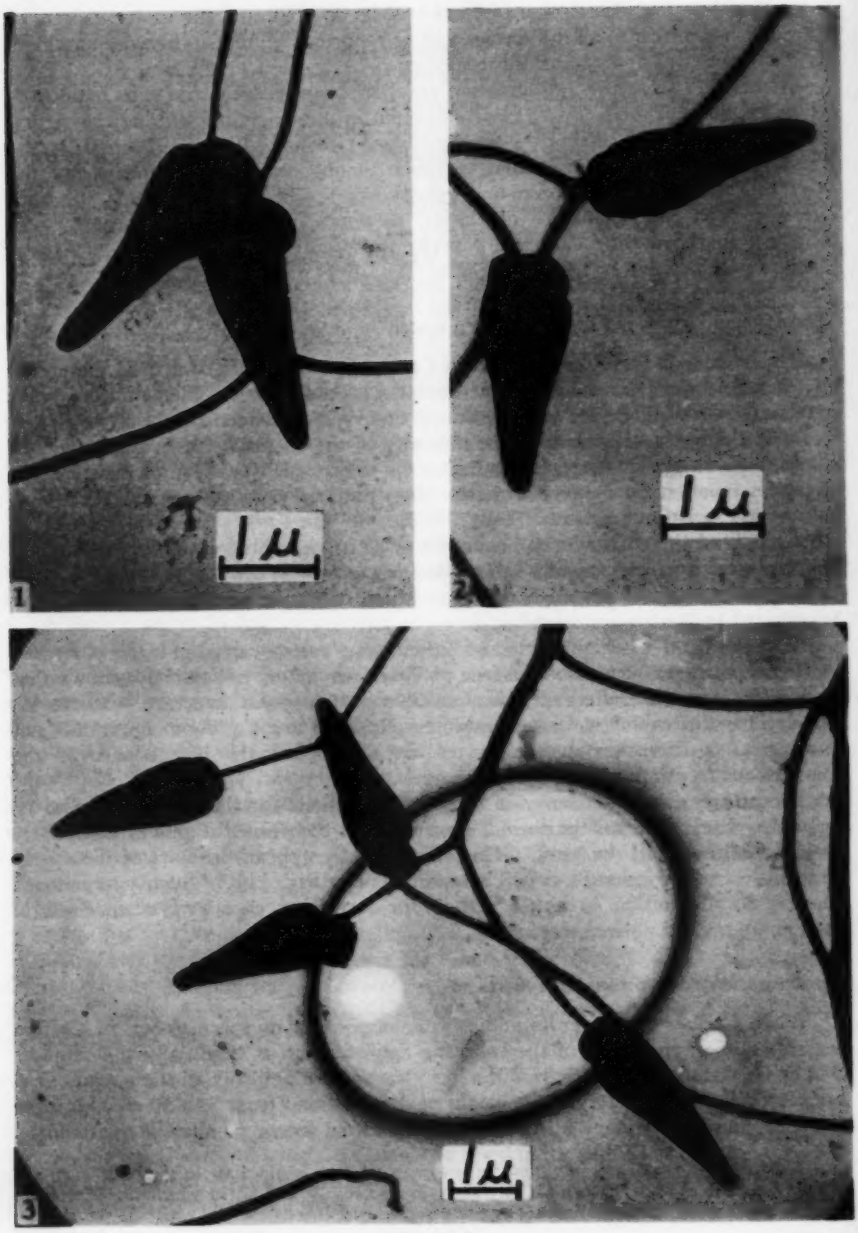
A comparison of Figures 1 and 2, electron micrographs of *Hemicentrotus* spermatozoa in sea water and Ca-free artificial sea water, respectively, shows that there is no immediate effect of lack of calcium on the sperm structure. Moreover, Figure 11 indicates that at least 10 minutes' exposure to a Ca-low medium does not produce any particular change in the appearance of the apical part of the spermatozoan (compare Fig. 8).

There is a marked tendency in Ca-low suspensions for the middle piece to be dislocated laterally from its normal position, and sometimes it moves completely around to the side of the head. This effect usually appears in most of the sperm after two or three minutes in a Ca-low medium (Fig. 11).[2] Such spermatozoa showed no diminution in either activity or fertilizing capacity (when used to inseminate eggs in normal sea water).

Agglutination reaction in Ca-low sea water

When Ca-low egg-water is added to a suspension of spermatozoa in Ca-free sea water, the appearance of the resulting agglutination is essentially the same as that in sea water, with the slight difference that while practically all the spermatozoa are involved in the clusters at the height of the reaction (from 5 to 30 seconds after the addition of egg-water) in sea water, a somewhat larger number of spermatozoa

[2] In this particular case only one spermatozoan shows a slight shift in the position of the middle piece; it appears much more strikingly in Figures 10 and 12, in which the effect may have been enhanced by the addition of egg-water (see Discussion).



FIGS. 1–3.

can be seen swimming freely between the clumps in Ca-low suspensions. In this medium, also, the suspended clumps are strikingly regular in shape, appearing to be perfectly spherical, in contrast to the more irregular masses formed in sea water agglutination.

One of the characteristics of reversible agglutination which is measurable to some extent is the duration of clumping. In all three of the species under consideration, it was found that, in general, a longer time was required in Ca-low than in sea water suspensions for reversal of agglutination. In *Hemicentrotus* this effect was only obtained with concentrated egg-water, such that in the sea water control a residue of small, permanently agglutinated clumps was left after the bulk of the suspension had returned to a free-swimming state. In *Anthocidaris,* Ca-low agglutination lasted 3–4 times as long as in the sea water controls, and in *Pseudocentrotus,* about twice as long.

Another quantitative comparison which can be made is that the effectiveness of a given solution of egg-water falls off much more rapidly with successive dilutions in the absence of calcium. For example, a lot of Ca-low egg-water which causes just perceptible (with low power microscopic observation of 1–2 cc. of suspension in a Syracuse watch-glass) agglutination of a Ca-low sperm suspension at a dilution of 1 : 64 may, after adjustment of its calcium content to that of normal sea water, cause an approximately equal agglutination of spermatozoa suspended in sea water at a dilution of 1 : 512.

In a sample of agglutinated sperm suspension in sea water, observed between slide and cover glass with high phase contrast magnification, there are practically no free-swimming spermatozoa, since the sperm which are not involved in the clumps are found held to one of the glass surfaces by their acrosome processes. In a sample taken after reversal of agglutination, nearly all the spermatozoa become attached to the glass, and the fact that their acrosomes have reacted is clearly observable. Electron microscopical observation of such suspensions after fixation shows all the spermatozoa with acrosome processes (Dan, 1952, Fig. 1).

In a similar sample in Ca-free sea water, however, all the sperm which are not held in the clumps are swimming freely, exactly as in Ca-low suspension before the addition of egg-water. Continuous observation of a small clump shows that while the total number of individuals in the aggregation does not noticeably increase or decrease, there is a constant coming and going of the peripheral members of the cluster. What appears to be the same behavior on a small scale is seen in certain more or less radial groupings of a few (5–10) spermatozoa at a time, at fixed points in the microscopic field (with the focus adjusted to the underside of the cover glass) where no object can be detected to form a center for such groups. The highly active spermatozoa abruptly join the group, their heads remaining more or less stationary for a few seconds or even minutes, although their tails are

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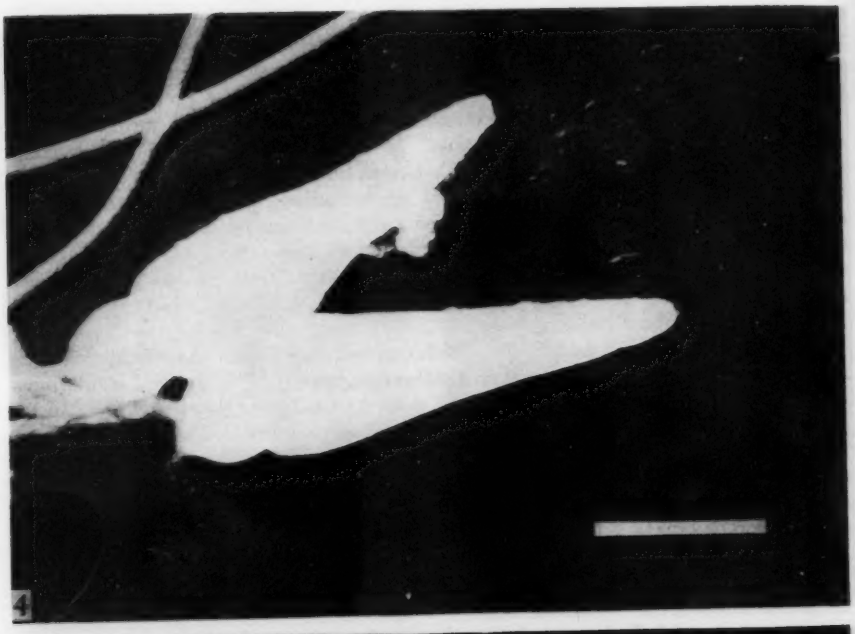
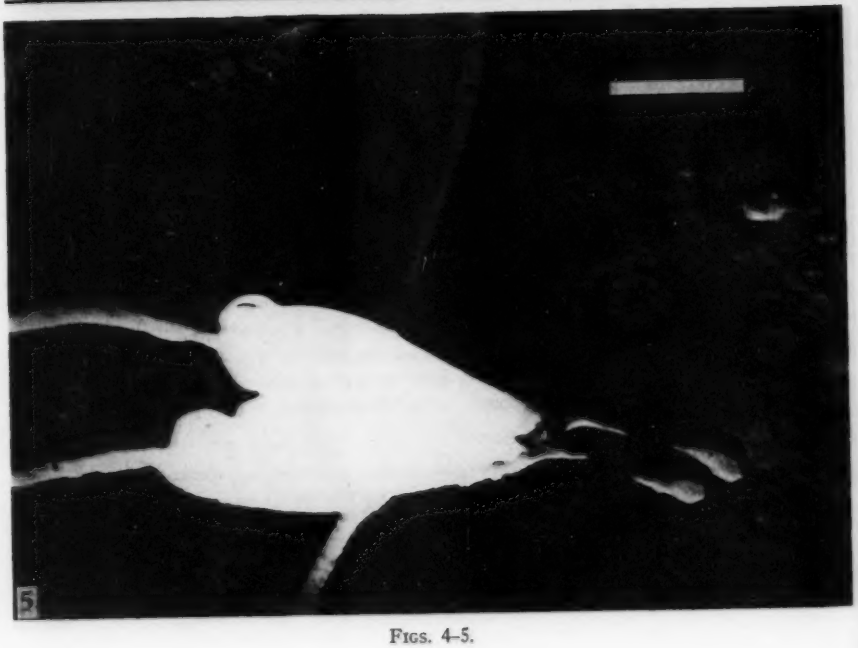
FIGURE 1. Electron micrograph of *Hemicentrotus pulcherrimus* spermatozoa, suspended for one minute in sea water and fixed with neutralized formalin.

FIGURE 2. *H. pulcherrimus* spermatozoa suspended in Ca-free artificial sea water for one minute, fixed with neutralized formalin.

FIGURE 3. Spermatozoa of *H. pulcherrimus* in Ca-free sea water, to which was added an equal amount of Ca-low egg-water. Fixed 30 seconds after the addition of egg-water, at the height of the agglutination reaction.





FIGS. 4–5.

lashing vigorously, and then they depart as suddenly as they arrived. In these small aggregates, as in the larger ones, the number of spermatozoa at any given time does not change materially. This behavior continues in the sample under the cover glass as long as the spermatozoa remain active, without respect to the reversal of agglutination in the main part of the suspension.

Acrosome reaction in Ca-low sea water

The easily observable fact that spermatozoa in a Ca-low medium do not adhere by their tips to the glass surfaces, even in the presence of egg-water, indicates that the acrosome reaction does not take place in such a medium. Phase contrast observation of individual acrosomes shows them all to be identical in appearance with those of freshly shed spermatozoa in pure sea water, and electron micrographs confirm this observation (Figs. 3, 10 and 12; compare with Figs. 1 and 8).

In order to differentiate between spermatozoa which had been involved in the clumps and those which had not, a strongly agglutinated suspension was hand-centrifuged to separate the clumped sperm from the freely swimming fraction. The sedimented clumps were re-suspended in fresh Ca-free sea water and examined for acrosome reaction. Both phase contrast and electron microscopic observation showed no difference between the acrosomes of these and of spermatozoa in the stock suspension (in Ca-low sea water before the addition of egg-water).

To control these observations, 0.36 *M* $CaCl_2$ was added to the Ca-low egg-water, making its calcium content equal to that of normal sea water (4%). This was then mixed with spermatozoa freshly suspended in sea water. Figures 4, 5, 6 and 7 show electron micrographs of spermatozoa of *H. pulcherrimus* fixed in pure sea water, immediately after the addition of egg-water, at the height of the agglutination reaction and after its reversal, respectively. Figures 8 and 9 show spermatozoa of *P. depressus* in sea water, and 30 seconds after the addition of egg-water.

Fertilizing capacity after agglutination in Ca-low medium

The fact has been repeatedly pointed out (Lillie, 1913; Tyler, 1941) that interaction with an agglutinating agent lowers the fertilizing capacity of a sperm suspension. The amount of diminution has been found to vary considerably from species to species and even among individuals within a given species, but in the writer's experience, also, there is always some reduction in fertilizing capacity following agglutination, when this takes place in a calcium-containing medium. It is equally clear, in the three sea urchin species under consideration, that there is no loss whatsoever in fertilizing capacity when the spermatozoa are agglutinated in Ca-low sea water. In this case, also, the clumped spermatozoa were quickly centrifuged out and re-suspended in fresh calcium-free medium, but no reduction in fertilizing capacity could be found after reversal of agglutination, either in these or in the

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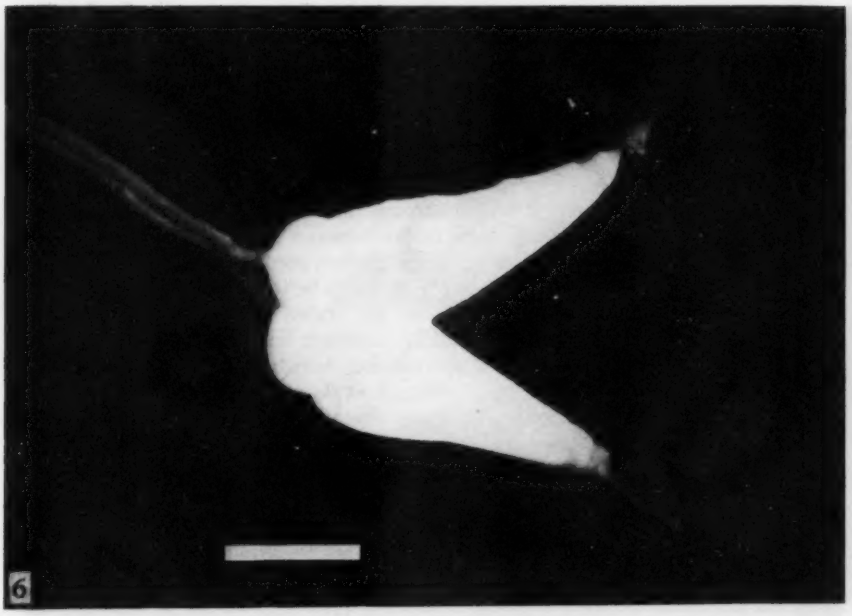
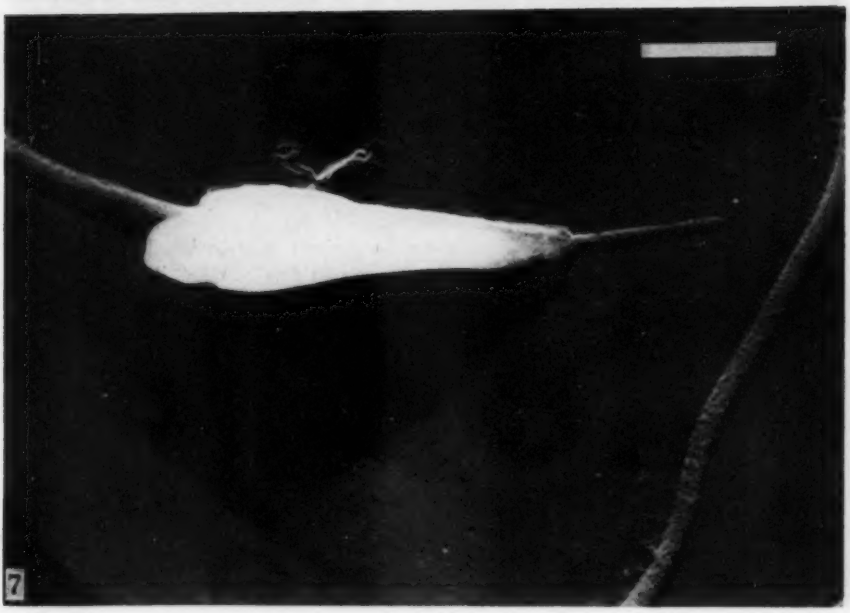
FIGURE 4. Spermatozoa of *H. pulcherrimus* in sea water, fixed with neutralized formalin. Chrome-shadowed electron micrograph.

FIGURE 5. *H. pulcherrimus.* Spermatozoa fixed two seconds after addition of homologous egg-water to sea water suspension. Breakage of acrosome filament commonly found in preparations fixed immediately after addition of egg-water (see Dan, 1952, Figs. 4 and 5).





FIGS. 6–7.

free-swimming fraction of the original suspension, when they were used to inseminate eggs in sea water.

In corresponding experiments with *H. pulcherrimus* spermatozoa agglutinated in sea water, it was found that while the supernatant fraction gave between 35 and 70% fertilization, similar dilutions of the clumped-reversed sperm gave between 0 and 3%.

Discussion

These results include a number of aspects for which no explanation is forthcoming at present. On the other hand, there are certain points which stand out with unexpected clarity. The most striking of these is the failure of a typical acrosome reaction when sea urchin spermatozoa are reversibly agglutinated by homologous egg-water in the virtual absence of calcium ions. This complete separation of the two reactions, which are normally elicited simultaneously by the one stimulus of egg-water, calls for a reconsideration of the problem in more detail.

An acrosome reaction to the stimulus of egg-water, which he thought provided an explanation for the reversible agglutination phenomenon, was first reported by Popa in 1927. Using Janus green-stained Arabacia sperm, Popa found that a sticky substance was produced at the tips of the sperm heads. He believed that this substance held the sperm together in clusters, and that its dispersion accounted for the reversal of agglutination. Improved optical apparatus has shown, however, that the substance (or structure) extruded from the sperm acrosome has apparently the same shape and adhesive properties long after the agglutination clusters have broken up (Dan, 1952). Popa's idea thus loses a good deal of its plausibility, since it fails to explain reversibility.

There are, however, more fundamental faults to be found in this scheme, since close observation of reversibly agglutinated spermatozoa clearly indicates, as Jacques Loeb (1914) reported forty years ago (p. 127), " . . . that the spermatozoa at the periphery of a cluster are in free progressive motion. When the clusters were small or when the sperm suspension was thin it was possible to observe the spermatozoa which are in the center of the cluster. It was seen that the spermatozoa in the center also were in very lively motion, with the possible exception of small lumps or groups of spermatozoa which may have stuck together. The clusters reminded the writer of a dense swarm of insects which move like a coherent mass through space. These clusters move like one solid body through the water, notwithstanding the fact that the individual spermatozoa are free to scatter."

This easily observable freedom of movement on the part of the agglutinated spermatozoa, as well as the fact that the regular clusters begin to be formed the moment egg-water is introduced into a sperm suspension and attain a large size within a few seconds by the fusion of smaller clumps, almost certainly rules out any possibility that the acrosome reaction can provide a physical basis for re-

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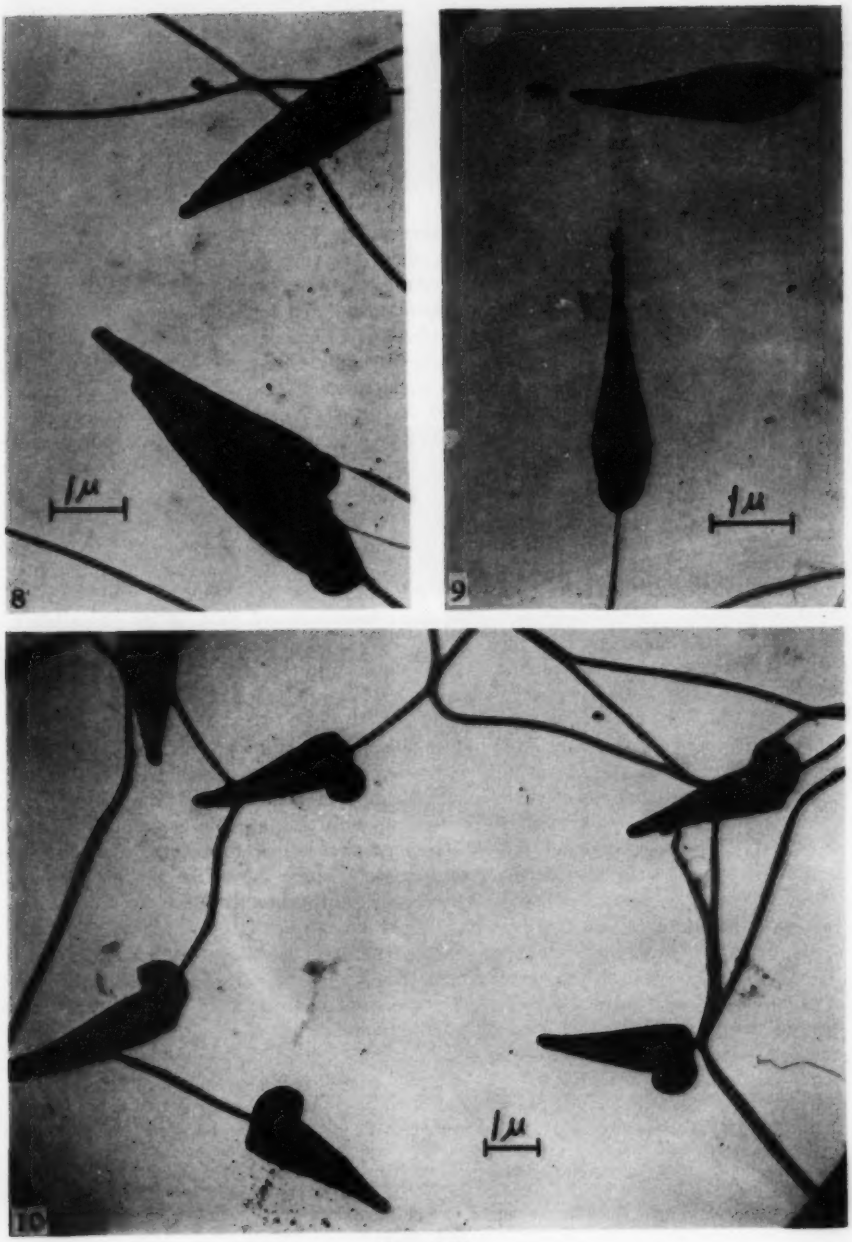
Figure 6. *H. pulcherrimus* spermatozoa fixed 20 seconds after acrosome reaction. The substance which was extruded has dissipated, exposing the acrosome filament.

Figure 7. *H. pulcherrimus* spermatozoan fixed three minutes after addition of egg-water, 30 seconds after reversal of agglutination. The acrosome filament has the same appearance as in the previous sample, fixed at the height of agglutination.



FIGS. 8–10.

versible agglutination. There is obviously some other force which instantaneously orients the swimming spermatozoa into groups and holds them there for a predetermined period, at the end of which they again become free to resume their independent behavior. Whether this is a tropistic reaction, as Loeb rather doubtfully suggested, a chemical reaction between fertilizin molecules and antifertilizin on the sperm head, as Tyler proposes, or some entirely different sort of effect, the writer does not know.

This negative conclusion, that the acrosome reaction cannot provide a causal explanation of the regular, oriented clustering found in reversible agglutination, is emphasized by comparison with the process of irreversible agglutination which follows exposure to hyperalkaline sea water, some foreign egg-waters, and various serum- or other protein-containing solutions. In these media the entire spermatozoan becomes sticky, and the cells adhere to each other at random, the whole suspension gradually becoming immobilized. As Loeb has described it (1914): " . . . the spermatozoa at first stick together to form short rows or threads; and later the threads begin to stick together to form irregular networks. At no time is there any appearance of cluster formation."

It is quite conceivable that small, regular clusters might be formed if each acrosome should become only sticky enough to adhere to another sticky acrosome,[3] but the time required for such cluster formation would greatly exceed the one or two seconds within which cluster formation is achieved in these species, and such an explanation does not provide at all for the addition of the second and succeeding layers of spermatozoa, or for the fusion of small clusters to form large ones.

A second kind of evidence dissociating agglutination and the acrosome reaction is found in the fact that sea urchin acrosomes react to the non-specific stimuli of contact with solid surfaces and hyperalkalinity, in neither of which reversible agglutination is involved.

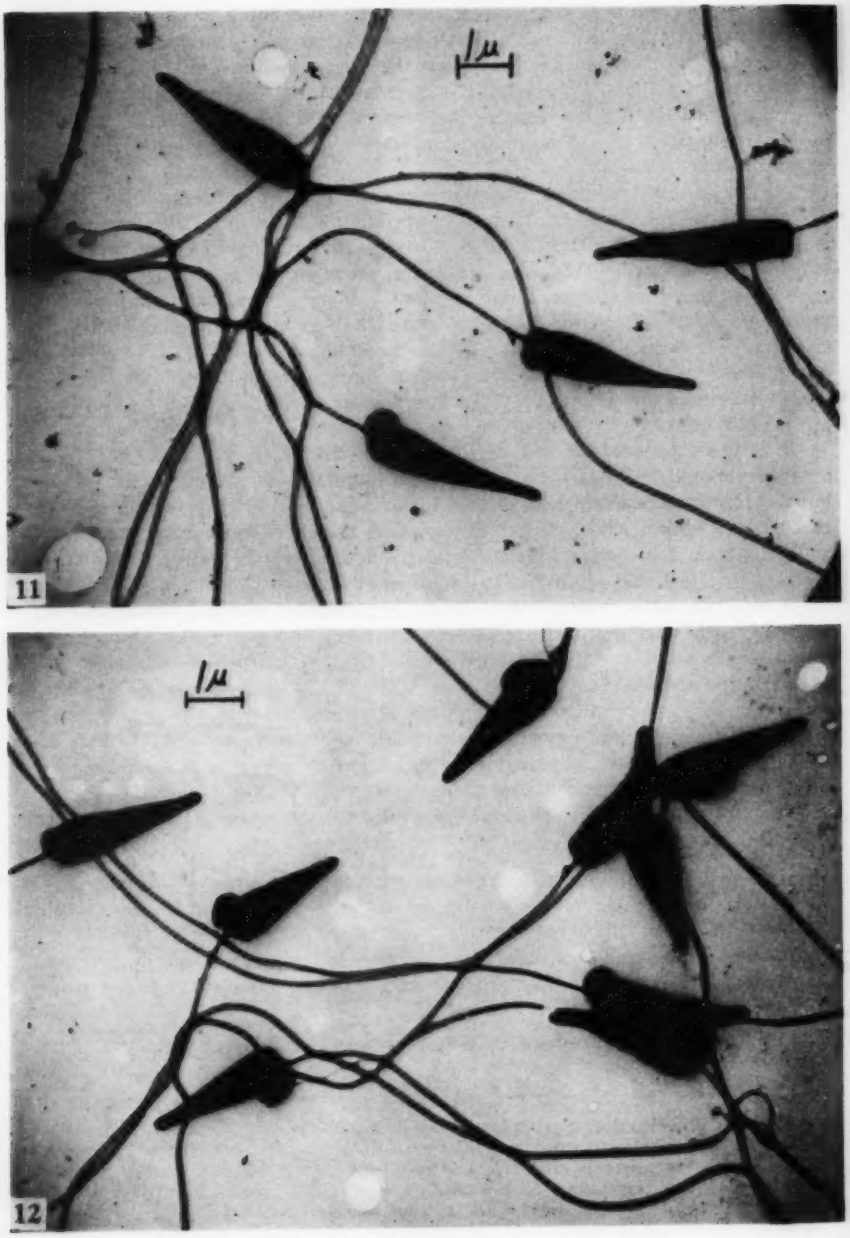
In the third place, there is the observation presented in this paper, that the minimum calcium requirement for reversible agglutination is very much lower than that for the acrosome reaction, so that it is possible to secure a typical agglutination reaction without any detectable response of the acrosomes.

Summarizing this evidence against a causal relation between the two reactions, we find that:

1. reversibly agglutinated spermatozoa are freely moving within the clusters, and therefore cannot be considered to be held together by any substance released from the acrosome;
2. there is no significant difference, in either living or fixed sperm, between the appearance of the acrosomes at the height of agglutination and after reversal;

[3] This may be the explanation of "rosettes" which are formed by the sperm of some echinoderms (*e.g., Mespilia globulus*) at the air-water and water-glass interfaces as a response to homologous egg-water.

FIGURE 8. Spermatozoa of *Pseudocentrotus depressus*, fixed after one minute in sea water.
FIGURE 9. Acrosome reaction of *P. depressus*, in response to homologous egg-water.
FIGURE 10. Spermatozoa of *P. depressus* suspended in sea water, and fixed 30 seconds after addition of Ca-low egg-water. Note lateral shift of middle pieces; this condition is also usually found in Ca-free suspensions before addition of egg-water.



FIGS. 11–12.

3. the clusters appear almost instantaneously after the addition of egg-water, which seems to preclude the possibility that they are formed by random acrosome-to-acrosome collisions. Moreover, this explanation would include only the smallest clusters;
4. the acrosomes react to stimuli which do not cause reversible agglutination, such as contact with solid objects and hyperalkalinity;
5. in the virtual absence of calcium, the stimulus of egg-water causes agglutination without any acrosome reaction.

Another, and perhaps more important, fact which emerges from the results of these experiments is that reversible agglutination without acrosome reaction has no effect on the fertilizing capacity of the spermatozoa. Lillie (1913) first showed that sperm suspensions which had been agglutinated by fertilizin had undergone a considerable (ca. 50%) reduction in fertilizing capacity. Tyler (1941), repeating Lillie's work, found a much greater loss of fertilizing capacity (p. 196): "Insemination with amounts of sperm that were well above the control minimum for 100 per cent fertilization gave in all tests with the agglutinated and reversed sperm between 0 and 3 per cent fertilization." [4] Rothschild (1947) has suggested that reversible agglutination may be (p. 241) "a form of false fertilization between spermatozoa instead of between eggs and spermatozoa; or at any rate, that there is some reaction between the surfaces of the spermatozoa which is sufficiently similar to that which occurs between an egg and a spermatozoon to alter the spermatozoon in an irreversible manner." Spermatozoa which have thus lost their fertilizing capacity following agglutination, Rothschild has characterized as being "muzzled" as the result of exposure to fertilizin in solution.

Among the three Misaki sea urchin species which undergo reversible agglutination, the writer has found considerable variation in the fertilizing capacity of reversed spermatozoa, especially if they are used for insemination immediately after reversal of agglutination. However, in every case there is a significant reduction in fertilizing capacity after agglutination in normal sea water, in which a majority of the acrosomes have reacted, and no reduction at all when agglutination has taken place in a calcium-low medium, where none of the acrosomes has reacted.

This result strongly suggests a causal linkage between the acrosome reaction and the fertilization reaction. Within a strict time limit, it appears that suspensions of spermatozoa with intact acrosomes possess full fertilizing capacity, while in those in which the acrosomes have reacted to such a stimulus as egg-water, this capacity is reduced. The suggestion fairly makes itself, that this acrosome reaction

[4] In these experiments, Tyler used very strong egg water, such that "agglutination usually lasted 30–40 minutes" (loc. cit.). Loeb (1914) gives 2–10 minutes as the range of duration of the agglutination reaction in the species used by Tyler (*Strongylocentrotus purpuratus*); the Misaki species also fall within this range. The possibility should be considered that egg-water strong enough to cause such extreme agglutination may have had a toxic effect on the spermatozoa.

FIGURE 11. Spermatozoa of *P. depressus* fixed 10 minutes after suspension in Ca-free sea water.

FIGURE 12. Spermatozoa of *Anthocidaris crassispina* suspended in Ca-free sea water and fixed one minute after addition of Ca-low egg-water, at height of agglutination reaction.

in response to fertilizin is the "false fertilization" of Rothschild, and its completion at a distance from the egg surface constitutes the muzzling effect on the spermatozoa.

The studies of Yamamoto (on fish eggs) and of Moser, cited above, have shown that the series of linked reactions constituting activation, the response of the egg, breaks down when calcium is excluded from the medium. The observations presented in this paper can be taken to indicate, however, that the stimulus itself is lacking, because of the failure of the acrosome reaction, when insemination takes place in a calcium-free medium.

Reference was made earlier to the change in position of the middle piece, which often occurs in all spermatozoa after a short exposure to plain Ca-low medium, without causing any diminution in the fertilizing capacity of the suspension. This same effect has been reported by Tyler (1952), who observed it in spermatozoa, suspended in sea water, which had been exposed to homologous egg-water. Tyler interprets this as an evidence of a general loosening of the sperm structure, preparatory to the complete separation of its parts within the egg cytoplasm. In this connection may be mentioned a similar relaxation of tension which occurs in starfish spermatozoa at the time of the acrosome reaction (Dan, 1954).

It is, moreover, almost certainly this same shifting of the middle piece under the influence of egg-water and various vital stains which was observed by Popa (1927) in *Arbacia* spermatozoa, although he described it as the formation of a "lateral body." However, his drawings show the middle piece tending to move around the basal end of the sperm head, and, significantly, his final figure representing the extruded "lateral body" at the side of the nucleus, shows a sperm head without any (other) middle piece.

Acknowledgment

It is a pleasure to acknowledge again the willing cooperation of the staff of the Misaki Marine Biological Station, and the kindness of Prof. A. Takamiya, of Tokyo Institute of Technology, who arranged for the use of the Institute's electron microscope. The photography was done by Mr. H. Akabori.

Summary

1. When sea urchin spermatozoa are suspended in Ca-free artificial sea water,

a) they show no morphological change, except that there is a tendency for the middle piece to become "loose" and move around to the side of the nucleus;
b) the sperm swim vigorously and attack the surface of unfertilized eggs as in normal sea water, but without being able to penetrate;
c) they do not undergo the acrosome reaction on contact with glass or collodion surfaces.

2. If "Ca-free" egg-water is added to such a sperm suspension,

a) a regular, reversible agglutination reaction occurs, which, with strong egg-water, lasts 2–4 times longer than in a corresponding suspension in which the calcium content is adjusted to that of normal sea water;

b) no acrosome reaction can be detected in spermatozoa thus agglutinated in the virtual absence of calcium;

c) after reversal of agglutination, such spermatozoa show no reduction in fertilizing capacity when used to inseminate unfertilized eggs in sea water. This is in marked contrast to the sea water control, in which there is a significant loss in fertilizing capacity following agglutination.

3. It is concluded that:

a) the agglutination reaction and the acrosome reaction must be considered to be separate phenomena, both occurring in response to the stimulus of egg-water, but not causally interrelated;

b) in view of the fact that fertilizing capacity is retained to the full extent by sperm suspensions in which the acrosomes remain intact, and is at least partially lost following mass acrosome reaction, this acrosome reaction must play an important role in the process of fertilization.

LITERATURE CITED

DAN, J. C., 1952. Studies on the acrosome. I. Reaction to egg-water and other stimuli. *Biol. Bull.*, **103**: 54–66.

DAN, J. C., 1954. Studies on the acrosome. II. Acrosome reaction in starfish spermatozoa. *Biol. Bull.*, **107**: 203–218.

GOLDFORB, A. J., 1929a. Variation of normal germ cells. Studies in agglutination. *Biol. Bull.*, **57**: 333–349.

GOLDFORB, A. J., 1929b. Changes in agglutination of ageing germ cells. *Biol. Bull.*, **57**: 350–388.

GOLDFORB, A. J., 1929c. Factors that change agglutinability of ageing sperm. *Biol. Bull.*, **57**: 389–411.

HEILBRUNN, L. V., 1937. An outline of general physiology. W. B. Saunders Co., Philadelphia.

KRAUSS, M., 1950. Lytic agents of the sperm of some marine animals. II. Extraction of a hetero-egg membrane lysin from sea-urchin sperm. *J. Exp. Zool.*, **114**: 279–292.

LILLIE, F. R., 1913. Studies of fertlization. V. The behavior of the spermatozoa of Nereis and Arbacia with special reference to egg extractives. *J. Exp. Zool.*, **14**: 515–574.

LOEB, J., 1914. Cluster formation of spermatozoa caused by specific substances from eggs. *J. Exp. Zool.*, **17**: 123–140.

LOEB, J., 1915. On the nature of the conditions which determine or prevent the entrance of the spermatozoon into the egg. *Amer. Nat.*, **49**: 257–285.

LOEB, J., 1916. The organism as a whole from a physicochemical viewpoint. G. P. Putnam's Sons, New York.

MOSER, F., 1939. Studies on a cortical layer response to stimulating agents in the Arbacia egg. II. Response to chemical and physical agents. *J. Exp. Zool.*, **80**: 447–471.

POPA, G. T., 1927. The distribution of substances in the spermatozoon (Arbacia and Nereis). *Biol. Bull.*, **52**: 238–257.

ROTHSCHILD, LORD, 1947. The problem of fertilization. *Brit. Med. J.*, **2**: 239–242.

SUGIYAMA, M., 1953. Physiological analysis of the cortical response of the sea urchin egg to stimulating agents. I. Response to sodium choleinate and wasp-venom. *Biol. Bull.*, **104**: 210–215.

TYLER, A., 1941. The role of fertilizin in fertilization of the eggs of the sea urchin and other animals. *Biol. Bull.*, **81**: 190–204.

TYLER, A., 1948. Fertilization and immunity. *Physiol. Rev.*, **28**: 180–219.

TYLER, A., 1952. Further investigations on fertilizins of eggs of sea-urchins. *Anat. Rec.*, **113**: 525–526.

YAMAMOTO, T., 1954. Physiological studies on fertilization and activation of fish eggs. V. The role of calcium ions in activation of Oryzias eggs. *Exp. Cell Res.*, **6**: 56–68.

ACTION OF AQUEOUS EXTRACT OF BEEF SPLEEN ON CELLS OF SARCOMA 37 ASCITES [1]

IRENE COREY DILLER, GEORGE F. WATSON [2] AND MARY E. FISHER

Institute for Cancer Research, Philadelphia 11, Pa. and Marine Biological Laboratory, Woods Hole, Mass.

An inhibitory action of beef spleen extract on growth of sarcoma 180 and mammary carcinoma of the mouse has been reported by Lewisohn (1938, 1945); of mouse sarcoma 37 and methylcholanthrene-induced sarcomas by Diller and Watson (1949); and of Brown-Pearce carcinoma of the rabbit by Nuttini *et al.* (1951). The mouse tumors studied by Diller and Watson (1949) showed degenerative cell changes resulting in shrunken bean-shaped nuclei and vacuolated cytoplasm at certain concentrations and dose levels, but no damage could be detected in normal tissues of the treated mice. MacFarlane and collaborators (1948) reported similar degenerative changes when cells of certain spontaneous tumors were stored in concentrated aqueous spleen extract for varying lengths of time at 5° C., which led them to believe that spleen extract could have a direct cytolytic action. More recently Katzberg (1952) incubated fragments of sarcoma 180 and of normal mouse tissues at 37° C. in Parker's medium and in medium containing either Rockland mouse spleen extract or Rockland mouse spleen pulp. Both malignant and non-malignant tissues remained viable in Parker's medium for ten days or longer. Splenic preparations apparently produced an agent which accelerated cytolysis. Maximum sensitivity to this agent was shown by sarcoma 180 (total destruction of cells in 48 hours) and maximum resistance by normal tissues of the Rockland mouse.

Our experiments (1949) with trocar-implanted or chemically induced tumors indicated that regression was accompanied by a marked inflammatory reaction in the vicinity of the tumor and an upsurge of mitotic activity in the organs of the hemopoietic system; a direct destructive action could not, therefore, be ascribed to the splenic extracts which we employed. Neither do such *in vitro* experiments as those of Katzberg (1952) offer clear-cut proof of direct cytolytic action, since under the conditions of his experiments, untreated tumor tissues, as well as normal tissue controls, were undergoing degeneration from the outset, and the effect of the extract, though more rapid in the case of tumor tissue, was largely an acceleration of degenerative change. The most convincing evidence of direct effect in Katzberg's experiments was the occurrence of metaphase block in sarcoma 180 during the first 24 hours after exposure to his splenic preparations.

In the hope that additional light could be thrown on the action of spleen extract in direct contact with tumor cells, further experiments were carried out in this laboratory using the ascites form of sarcoma 37, the solid form of which had been studied previously in this connection (Diller and Watson, 1949).

[1] Supported in part by an institutional grant from the American Cancer Society and in part by a grant from the Eli Lilly Company.

[2] Kitchener Clinic, Kitchener, Ontario, Canada.

Material and Methods

Albino mice, 6 to 8 weeks of age, both males and females, of the special Swiss strain maintained in the main colony of our Institute were inoculated intraperitoneally with one cc. of sarcoma 37 ascites tumor. The growth was allowed to develop for three days, when abdominal swelling could be observed visually. A sample of the ascites fluid (see Fig. 1) was withdrawn, diluted 1:200 in saline solution and counted in a hemacytometer. The animals were then injected intraperitoneally with a single dose (one cc.) or with 4 doses (0.25 cc. each) of varying concentrations (50 mg., 100 mg., and 150 mg. of solids/ml.) of crude aqueous cell-free, beef spleen extracts (not commercially available) obtained through the courtesy of the Eli Lilly Company, or prepared at the Kitchener Clinic and processed in Philadelphia by the McNeil Laboratories.[3] In the Kitchener Clinic the extract is prepared by mincing 400 grams of fresh calves' spleen under sterile conditions and extracting it in 1000 cc. of sterile saline at 33°–35° C. Approximately 400 cc. of the supernatant is decanted after four weeks and passed through a Seitz filter. Details of preparation and clinical use will be treated in a forthcoming paper under the authorship of the medical member of the group (G. F. W.). Control animals received corresponding amounts of sterile salt solution. The extracts were found to be very unstable, even though prepared and maintained constantly at temperatures just above freezing. Color change from red to dark brown usually accompanied loss of potency.

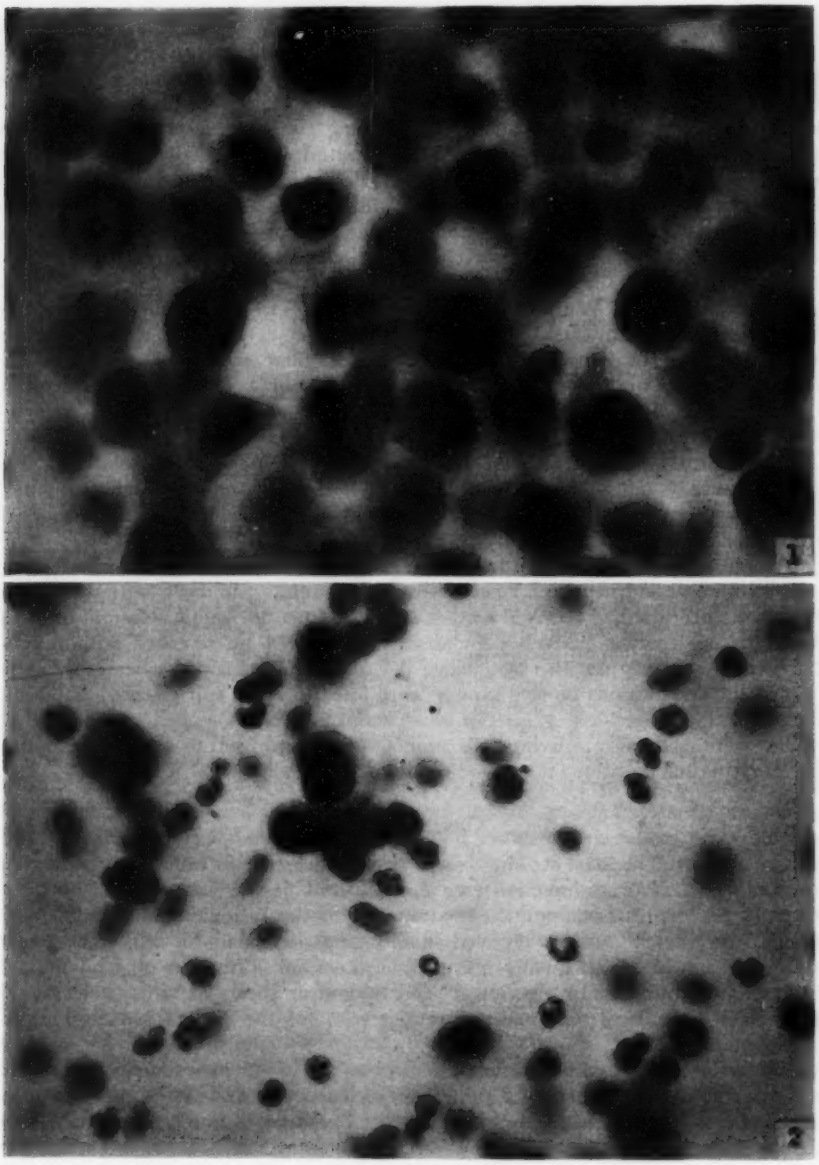
Ascitic fluid was withdrawn from each mouse at intervals of 6, 24 and 48 hours after spleen extract injection for hemacytometer counts. Similar counts were made of control mice that had received neither spleen extract nor saline solution, in order to determine whether the cell population was affected by repeated withdrawals. This was apparently not the case, since the cell count remained essentially static. Permanent preparations of each sample were fixed in Allen's or Carnoy's fluid and stained with Feulgen-fast green for differential counts. Detailed phase contrast studies were made also of living cells stained with neutral red to aid in detection of degenerating nuclei, after the method of Goldie and Felix (1951).

Observations

Cell count. The most striking result was the decrease in tumor cell population following spleen extract injection (see Table I). This was accompanied by subsidence of the ascites swelling. Stained smears of the residual fluid (usually less than 0.5 cc. after 48 hours) revealed small clumps or islands of cells as indicated in Figure 3, which presumably served as centers for formation of solid masses. Some control animals also developed solid tumors or sheets of tumor cells on the peritoneum, but there was never any regression of the ascites swelling in untreated mice.

The reduction in number of tumor cells observed in our experiments was apparently in part the consequence of infiltration of leucocytes and histiocytes, by which the tumor cells were phagocytized, in part of cessation of mitosis, and in part of cellular degeneration (see table of differential counts).

[3] The authors wish to thank Dr. Paul N. Harris of the Eli Lilly Company and Mr. Robert McNeil, Jr., of the McNeil Laboratories, for their interest and assistance.



FIGS. 1–2.

Sarcoma 37 ascites tumor preparations from mice injected with aqueous beef spleen extract. Picric-formol-acetic fixation, Feulgen-fast green.

TABLE I

Cell count per cc. of sarcoma 37 ascites fluid (Average of 20 animals)

	Spleen inj. One cc. of 150 mg. conc.	Saline inj. One cc.	Uninjected
Pre-inj.	50.2×10^6	54.0×10^6	54.9×10^6
6 hrs. post-inject.	30.2×10^6	53.5×10^6	54.5×10^6
24 hrs. post-inject.	21.2×10^6	50.3×10^6	53.7×10^6
48 hrs. post-inject.	26.0×10^6	51.7×10^6	51.2×10^6

Mitotic phenomena. Counts of 1000 cells per specimen were made on stained preparations of fluid withdrawn prior to spleen extract injection, and at 6 hours, 24 hours, 48 hours and 72 hours post-injection. An average of 3.6% of the tumor cells were in mitosis just before treatment. In many individuals no mitotic figures at all could be observed 6–24 hours after injection of spleen extract, and in all individuals there was a decreased percentage of cells in mitosis (see Table II). When one cc. of spleen extract (150 mg. of solid/cc.) was administered in a single dose, mitotic activity was suppressed for at least 48 hours, but if smaller doses of extract were given, it was necessary to repeat them at 6-hour intervals in order to achieve this result.

TABLE II

Differential count, based on 1000 cells per mouse per sample*

	Mitosis %				Necrosis %			Polymorphs %				Lymphs %				Tumor cells (non-dividing) %			
Treatment:	Pre	6 h	24 h	48 h	Pre	6 h	48 h	Pre	6 h	24 h	48 h	Pre	6 h	24 h	48 h	Pre	6 h	24 h	48 h
Spleen extract 50 mg./ml.	3.1	0.3	0.0	0.2	0.4	1.5	3.8	17.5	39.8	59.7	32.4	3.0	10.0	13.6	24.0	76.0	48.4	26.7	39.6
Spleen extract 150 mg./ml.	3.7	1.4	1.4	0.1	0.4	0.4	60.1	20.9	28.8	40.0	24.9	4.4	4.3	6.0	9.4	70.6	65.1	52.6	5.5
Saline inj.	3.9	3.5	3.6	3.7	1.1	0.2	1.5	20.3	16.8	18.1	19.4	8.5	7.5	9.7	6.1	66.2	72.0	68.6	69.3
Non-inj. controls	3.6	3.4	3.8	3.8	1.3	1.2	2.9	13.0	13.9	18.9	16.2	9.5	10.2	9.9	10.8	72.6	71.3	68.3	66.3

* 20 mice in each group.

Blood cell counts.

a. *In peritoneal fluid.* The number of polymorphonuclear leucocytes increased immediately after spleen injection in all cases (Fig. 2), and continued to rise for about 30 hours, then gradually returned to the level of the controls as shown in Table II. There was an accompanying increase in percentage of lymphocytes, which persisted into the 48-hour period. The lymphocytic count agrees with that

FIGURE 1. Smear preparation of untreated control, three days after inoculation into the peritoneal cavity. Ca. 800 ×.

FIGURE 2. Smear preparation of ascitic fluid withdrawn from the same mouse 24 hours after intraperitoneal injection of spleen extract (50 mg./ml.). Only two tumor cells remain in a comparable field; the remainder of the cell population is made up of leucocytes. Ca. 800 ×.

observed when solid tumors were treated with spleen extract as described in an earlier paper (Diller and Watson, 1949). Macrophages and other histiocytes were present also in the ascitic fluid, but there was no significant change in number of these cells following spleen injection, as was the case with solid tumors (Diller and Watson, 1949; Lewisohn, 1938, 1945).

b. *Peripheral blood.* A comparison of the effect of injection of spleen extract on the peripheral blood picture (tail vein samples) of tumor-bearing and non-tumor-bearing mice appears in Table III.

TABLE III

Peripheral (tail vein) leucocyte count (Average of 20 mice)

Mouse strain	Tumor- or non-tumor-bearing	Treatment	Route of inj.	Duration of treatment	W.B.C. count/mm.³ 1:20 dil.
Swiss	Non-tumor	—	—	—	15,459
Swiss	Non-tumor	Spleen ext. 150 mg./cc. 1 cc. daily.	I.P.	6 days	15,740
Swiss	S-37 solid 7 days post-implant.	—	—	—	21,990*
Swiss	S-37 solid 7 days post-implant	Spleen ext. 100 mg./cc. 1 cc. daily beg. 4th day post-implant.	I.P.	24 hrs. 72 hrs.	30,060 32,530
Swiss	S-37 ascites 5 days post-implant.	—	—	—	18,500
	S-37 ascites 5 days post-implant.	Spleen ext. 150 mg./cc. 1 cc. on 3rd day post-implant.	I.P.	48 hrs.	39,120

* Data from an earlier experiment (1949). Fekete in *Biology of the Laboratory Mouse*, 1941, p. 94, reported an average peripheral count (based on unpublished data of Law and Heston) of 21,510 for tumor-bearing Bagg albinos.

Percentage of viable tumor cells. The percentage of non-dividing tumor cells before and after injection, based on a count of 1000 cells per preparation, is also shown in Table II. Animals injected with the Lilly extract showed an average decrease in number of tumor cells from 76% before injection to 26.7% at 24 hours. This was apparently due to several factors—dispersion of cells by reason of infiltration of leucocytes, cessation of mitosis, and, as indicated by uninjected controls, to slight lowering of cell numbers by daily withdrawal of fluid. With the fall of the leucocyte count at 48 hours and resumption of mitosis, the tumor cell count per 1000 cells was increased to 39.6%. Following the injection of an extract

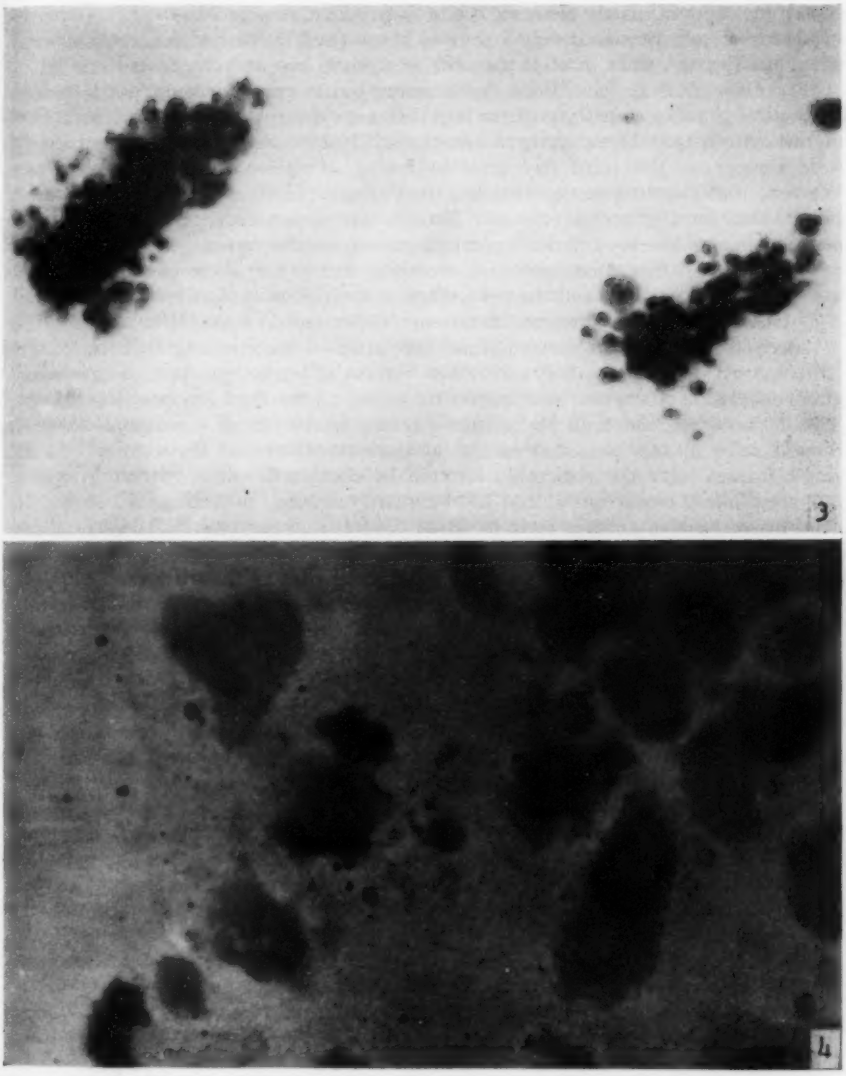


FIGURE 3. Smear preparation of ascitic fluid from the same mouse 72 hours after intraperitoneal injection of spleen extract. Small solid tumors are beginning to form around islands made up of aggregations of tumor cells and leucocytes. Ca. 400 ×.

FIGURE 4. Smear preparation of ascitic fluid from a mouse 48 hours after intraperitoneal injection of spleen extract (150 mg./ml.) showing total degeneration of almost all tumor cells. (In such cases only about 5% of the tumor cells remained viable.) Ca. 1100 ×.

containing approximately 150 mg. of solids/ml., the percentage of apparently viable tumor cells remained very low at 48 hours (only 5.5% had a normal appearance; see Fig. 4), since most of the cells were shrunken and coagulated.

Morphological changes. The degenerating tumor cells just mentioned showed cytological changes consisting of nuclear shrinkage and pycnosis and vacuolization of the cytoplasm. These changes are essentially the same as those observed in solid tumors on the third day after beginning of spleen injection (Diller and Watson, 1949) and those reported by MacFarlane (1948) after storage of tumor cells in concentrated spleen extract. No evidence of interference with the spindle mechanism was observed in our experiments with ascites tumors, though Katzberg (1952) reported this phenomenon as occurring in the first 24 hours of storage in his splenic preparations, and the percentage of multipolarity was increased in solid S-37 treated by us with low concentrations (Diller and Watson, 1949).

Survival. Maximum survival time was attained by injecting 0.25 cc. of the Lilly extract four times daily at 6-hour intervals, beginning either immediately after inoculation of the host with ascites tumor, or on the third day post-inoculation. This induced regression of the ascites swelling in 16 out of 60 animals treated, though solid masses persisted in the abdominal cavities of these mice. Large single masses were not noticeably affected by continued spleen extract injection, but grew like a trocar-introduced solid tumor, resulting in death of 10 of the 16 solid-tumor-bearing animals in from 18–24 days (mean survival 20.7 days). Control animals bearing untreated ascites tumors died 7–9 days (mean 7.1 days) after inoculation. In the group of 16 mice in which solid tumors developed, complete resorption of smaller masses was induced in 6 animals under continued spleen therapy. The host animals were still tumor-free six months later, when they were sacrificed. It will thus be seen that even the most effective samples of extract with respect to mitotic inhibition and tumor cell destruction did not improve the chance of survival of the host unless the ascitic fluid was absorbed and the neoplasm converted to the solid form. Of these solid tumors, about one-third regressed under continued spleen extract injection. Since the fate of small solid sarcoma 37 in mice treated with similar spleen extracts is already known (Diller and Watson, 1949) further studies on larger numbers of animals were not carried out.

In most of our experiments ascites swelling was already visible when treatment was begun. This procedure was followed in order to ensure actual presence of neoplasia. However, studies were made also on the effect of injecting the extract prior to inoculation with ascites fluid. If no further spleen extract injections were made after inoculation of the tumor, the course of development was unaffected. On the other hand, when injection of the extract was resumed on the day of inoculation of the tumor, all the animals died with hemorrhage in the peritoneal cavity before ascites swelling appeared. Non-tumor-bearing control mice suffered no casualties when subjected at the same time intervals to interrupted dosage of the same magnitude. Simultaneous injection of extract into the peritoneal cavity of mice at the time of tumor inoculation did not prevent the development of the ascites tumor.

Discussion

There are two types of reaction to injection of the splenic extract into mice bearing sarcoma 37 ascites:

1. *An inflammatory one* involving a sharp increase in the number of polymorphonuclear leucocytes (Fig. 2) and a less marked, but more persistent, increase in the number of lymphocytes present in the ascitic fluid. The same phenomena were observed in the case of mice bearing solid sarcoma 37 (Diller and Watson, 1949), since on the third day after beginning of treatment there was considerable infiltration of the tumor tissue by leucocytes, including an increased number of macrophages.

2. *Inhibition of mitosis* in all of the tumors and varying amounts of necrotic change (depending on the sample of spleen extract employed) with consequent reduction in tumor cell population accompanied by formation of small solid tumors.

In addition to reports of spleen-extract-induced regression of already developed tumors, noted at the beginning of this article, there have been numerous accounts of the inhibitory effect of spleen extract on establishment of tumor transplants or on experimental tumor induction. For example, Sugiura (1938) found that aqueous extracts of several organs of the rat had a depressant action on growth of mouse sarcoma 180 and on Flexner-Jobling rat carcinoma when, prior to implantation, the tumor fragments were stored in the extracts at 4–5° C. before implantation into mice. He reported also cessation of mitosis *in vitro* with degenerative nuclear changes under the same experimental conditions.

Yun (1950a, 1950b, 1950c) reported that pre-injection of mice with splenic extract inhibited development of tumors in mice painted with methylcholanthrene and also that extirpation of the spleen from A strain mice furthered the development of methylcholanthrene tumors.

When Nuttini (1951) pre-injected rabbits with rabbit spleen pulp or cell-free filtrate thereof, 66% of the host animals were protected against development of Brown-Pearce carcinoma. Therapeutic administration of the filtrate also induced total regression in a significant percentage of the animals treated.

In 1952 Foley reported retardation and/or regression of lymphosarcoma in C3H mice implanted subcutaneously with cell suspensions of spleen from an alien mouse strain. Also according to Lorenz *et al.* (1953), spleen shielding and bone marrow injections effectively protect mice against x-ray induction of lymphoid tumors. When C57 Black mice were given four doses of 225 r without spleen protection 70% of the mice developed lymphoid tumors but after the same amount of irradiation in a spleen-shielded group tumors appeared in only 3% of the animals. On the basis of studies of tumor tissue and spleen tissue grown *in vitro* Pollard and Bussell (1953) showed that spleen tissue from the host asserted no destructive effect on its own tumor tissue though similar tissue from the spleen of a rat that had sloughed a tumor had a rapid destructive effect.

That the inhibition of mitotic processes is not confined to neoplastic tissue in contact with splenic extract was indicated by Fardon *et al.* (1948) who reported partial inhibition of mitosis *in vivo* in the crypts of Lieberkühn of mice injected with beef spleen extract; and Hoffman (1940) found that extract of kidney, spleen, liver and lung definitely inhibited growth of fibroblast colonies *in vitro*.

Rohdenburg and Nagy (1937) showed that *both* inhibitory and stimulatory materials are present in rabbit and human spleen. The inhibitory agent was found in the initial acetone-soluble fraction. In our earlier experiments (1949) spleen, liver, thymus, lymph nodes and, occasionally, adrenal tissues of the treated mice were stimulated to mitotic activity by doses that were capable of causing regression

of solid tumors. This led us at that time to postulate that the suppressive effect on tumors was probably due, not to direct cytolysis, but to stimulation of the hemopoietic organs and the adrenal cortex. Furthermore, extract of low concentration appeared to stimulate mitosis in solid tumors, resulting in polyploid cells (as judged by numbers of nucleoli) and multipolar spindles. It is possible that these were, in fact, damaged cells which would eventually have become inviable. Bearing on this point are the findings with respect to ascites tumors injected with the 50 mg./ml. concentration (Table II) where inhibition of mitosis was obtained more rapidly with the low than with the high concentration. However, the 150 mg./ml. preparation produced drastic cell damage and eventual destruction of a much higher percentage of tumor cells (only 5.5% viable after 48 hours) though the host animals usually did not survive more than an additional day and died ahead of the controls. Non-tumor-bearing control mice tolerated injections of the same magnitude without adverse reaction, which suggests that toxic substances were being released by the degenerating cells. Klein (1951) reported that if cells are damaged before or after introduction into the peritoneal cavity, the course of events will be the same as though low cell numbers were originally injected, *i.e.*, there occurs a very intense inflammatory reaction after which tumor cells disappear completely from the peritoneal fluid, or are present in relatively low numbers and the animals die much later with solid tumors. Apparently this is what occurred in our own experiments in 10% of the animals.

Cook and collaborators (1951) attempted to determine the mechanism by which tissue extracts operate in growth processes and reported that spleen extract has an inhibiting effect on certain enzyme systems known to function in cellular respiration. Aqueous homologous cell-free spleen preparations were the most effective in stimulating oxygen uptake. Water-soluble spleen extract caused depression in various concentrations, except those from 25–10 mg./ml., which produced stimulation. In general, neoplastic tissues showed greater changes than did normal tissues.

Whether or not splenic extract exerts a direct cytolytic action on tumor cells is still not proven since the evidence from ascites tumors is that the host participates through leucocytic response. However, the experiments herein described or reviewed appear to indicate that there are substances present in splenic extract capable of suppressing mitosis for varying periods of time and of inducing cytolysis in tumors, while at the same time stimulating the hemopoietic system of the host.

Summary

1. The injection of a single dose of aqueous beef spleen extract containing 50 mg.–150 mg./ml. of solid materials directly into the peritoneal cavity of mice bearing ascites sarcoma 37 resulted in inhibition of mitosis for a period of at least 48 hours, and in some cases in complete cessation of mitosis that persisted for even longer periods. Mitotic inhibition was accompanied by a marked inflammatory reaction. Cell counts per cc. of ascites fluid were lowered and the percentage of viable tumor cells decreased, partly as the result of cessation of mitosis and necrotic change, and partly through dilution with white blood cells.

2. The reduction of tumor cell population was accompanied by the aggregation of tumor cells into islands which presumably served as centers for the formation of

solid tumors. When the tumor masses thus formed were large, no regression was observed, though survival time was considerably lengthened; however, small masses were completely resorbed under continued spleen extract therapy in a third of the animals whose tumors had become solid under spleen extract injection (10% of the total number of animals treated). No reduction in ascites swelling unaccompanied by the formation of solid tumors was observed.

LITERATURE CITED

COOK, ELTON S., J. C. FARDON AND SISTER JEANNE DE LORRAINE, 1951. A comparative study of the action of spleen preparations on the respiration of normal and neoplastic tissues. *Acta Unio int. contra Cancrum*, **7**: 552.

DILLER, I. C., AND G. F. WATSON, 1949. Effect of aqueous spleen extract on growth of tumor cells in mice. *Growth*, **13**: 27–44.

FARDON, J. C., J. E. PRINCE AND N. BERNING, 1948. Effect of beef spleen extract on mitosis in the small intestine of the mouse. *Cancer Res.*, **8**: 531.

FOLEY, E. J., 1952. Resistance of C3H mice to lymphosarcoma 6-C3H-ED induced by tissues from mice of C3H sublines. *Proc. Soc. Exp. Biol. Med.*, **81**: 671–674.

GOLDIE, HORACE, AND MARIE DINGMAN FELIX, 1951. Growth characteristics of free tumor cells transferred serially in the peritoneal fluid of the mouse. *Cancer Res.*, **11**: 73–80.

HOFFMAN, R. S., 1940. The growth-activating effect of extracts of adult and embryonic tissue of rats on fibroblast colonies *in vitro*. *Growth*, **4**: 361–376.

KATZBERG, ALLAN A., 1952. Tissue lysis by splenic preparations. *Growth*, **16**: 15–25.

KLEIN, GEORG, 1951. The production of ascites tumors in mice and their use in studies on some biological and chemical characteristics of neoplastic cells. Almqvist and Wirksells Boktryckeri, Uppsala.

LEWISOHN, R., 1938. Effect of subcutaneous injections of concentrated spleen extract on mouse sarcoma 180. *Surg. Gyn. Obstet.*, **66**: 563–576.

LEWISOHN, R., 1945. Recent advances in cancer research. *J. Mt. Sinai Hosp.*, **12**: 476–479.

LORENZ, E., C. C. CONGDON AND DELTA UPHOFF, 1953. Prevention of irradiation-induced lymphoid tumor in C57B1 mice by spleen protection. *Proc. Amer. Assoc. Canc. Res.*, **1**: 34.

MACFARLANE, E. W., N. G. SCHMUCK AND L. V. NADEAU, 1948. Effect of cold and of beef spleen extract on dbrB mouse tumor cells as shown by growth of transplants into dba mice and by cytologic examination. *Cancer Res.*, **8**: 438–448.

NUTTINI, LEO G., DOROTHY LETANG AND FRANK X. BIONDO, 1951. Effect of homologous and heterologous splenic materials on the incidence and regression of the Brown-Pearce carcinoma in rabbits. *Acta Unio int. contra Cancrum*, **7**: 794.

POLLARD, MORRIS, AND ROBERT BUSSELL, 1953. Role of the spleen in resistance to experimental tumors. *Texas Rept. Biol. Med.*, **11**: 48–58.

ROHDENBERG, G. L., AND A. M. NAGY, 1937. Some further physiological effects of cell division stimulants and inhibitors. *Amer. J. Canc.*, **30**: 512–516.

SUGIURA, K., 1938. The effect of extracts of different organs and tissues upon the viability of transplantable tumors. *Amer. J. Canc.*, **32**: 126.

YUN, IL SUN, 1950a. Influence of the spleen on the induction of tumors in mice with subcutaneously injected 20-Methylcholanthrene. Abst. in *Proc. of Fifth Int. Cancer Congress*, Paris.

YUN, IL SUN, 1950b. The effect of the spleen on the production of hepatic tumors in animals following the introduction of 20-Methylcholanthrene into the liver. Abst., *loc. cit.*

YUN, IL SUN, 1950c. Effects of splenic extract injection on the development of tumors in the skin of mice by 20-Methylcholanthrene. Abst. *loc. cit.*

THE EFFECTS OF TEMPERATURE ON THE SUCROSE THRESHOLDS OF THE TARSAL CHEMORECEPTORS OF THE FLESH FLY, SARCOPHAGA BULLATA [1]

HUBERT FRINGS AND BEVERLEY L. COX

Department of Zoology and Entomology,
The Pennsylvania State University, State College, Pa.

It has long been known that insects possess contact chemical receptors which enable them to select desirable and reject undesirable foods. Earlier studies on the locations of these receptors are reviewed by Frings and Frings (1949) and on the functions by Dethier and Chadwick (1948). One of the many problems evolved from these studies is whether stimulation of gustatory receptors is due to absorption, in which the stimulating substance penetrates the exoskeleton, or to adsorption, in which the substance adheres to the surface of the taste receptor. Dethier (1951) suggests that penetration is most important by stating that the limiting mechanism in tarsal chemoreception probably involves a two-phase system, in which highly water-soluble compounds gain access to the receptor through an aqueous phase and the larger lipoid-soluble molecules chiefly through a lipoid phase.

A likely hypothesis, if penetration is involved, would be that the higher the temperature, within limits of death, the faster the penetration and the lower the threshold. Conversely, the lower the temperature, the slower the penetration and the higher the threshold. If, on the other hand, adsorption is involved, the negative temperature coefficient, other factors being equal, would cause the threshold to be lower at lower temperatures and higher at higher temperatures. The present study was an attempt to answer this absorption-adsorption question by testing the effects of temperature on the sucrose threshold of the tarsal chemoreceptors of the flesh fly, *Sarcophaga bullata.*

Materials and Methods

The flies were reared in the laboratory as described by Frings and Frings (1952) and Knipe and Frings (1952). Adults were kept in wire cages (30 × 30 × 30 cm.), where they were given a constant supply of water and sugar cubes. Protein was supplied to the flies in the form of skinned dead mice. A mouse was supplied every 24 hours to each cage of flies until larviposition occurred, which was within 5–7 days after the protein was first furnished. After approximately 200 larvae were obtained, the mice were withheld until a new supply of larvae was needed. This insured a constant supply of larvae and flies at regular intervals. The larvae were reared on moistened dog biscuit.

Techniques described by Frings (1947) were used in mounting the flies for

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testing. After they had recovered from mounting, each was allowed to consume to satiety water and 1.0 *M* sucrose solution.

The constant temperature chamber used in this study consisted of two plywood boxes, one within the other. The inside box (56 × 56 × 56 cm.) was separated from the outer one (64 × 64 × 64 cm.) by 4 cm. of insulating material. Both chambers had removable tops with glass apertures (30 × 30 cm.) situated in the center to provide necessary illumination for the inner chamber, which was easily viewed through glass doors (30 × 30 cm.) at the front of the boxes. To aid in the maintenance of desired temperatures, a suction fan was installed near the top of the inner chamber to circulate the air after it was heated by an infra-red bulb or cooled by ice. Ventilation of the chamber was controlled by a sliding glass panel made to cover an opening (6 cm. diam.) situated directly in front of the fan. A light socket was installed near the bottom of the inner chamber for the infra-red bulb and the electrical apparatus of the light and fan were operated from the outside. A slit (5 × 30 cm.) was cut along one side of the boxes for the purpose of inserting the experimental animals. This opening was covered on the outside with compressed sponge rubber with corresponding slits through which the mounted flies could be inserted. A table (56 cm. long × 15 cm. wide × 15 cm. high) was placed in the chamber to hold Syracuse watch-glasses containing the test solutions.

Much preliminary experimental testing with sucrose molarities was done before settling on the following molarities used throughout the experiment: 0.02 *M*, 0.04 *M*, 0.06 *M*, 0.08 *M*, 0.1 *M*, 0.2 *M*, 0.4 *M*, 0.6 *M*, 0.8 *M*, and 1 *M*. A fresh stock solution (1.0 *M*) was made up every four or five days and refrigerated between tests to avoid fermentation. Other molarities were made up from the stock solution every two days.

The flies were tested in the constant temperature chamber at three ranges of temperature: 19–21° C., 27–29° C., and 37–39° C. The experimental temperature for each day was picked at random in order to avoid the flies' possibly learning a particular pattern of testing. The temperatures, 27–29° C., were those of the box without the addition of heat or ice. A pan of water in the chamber assured a saturated atmosphere in the box. For temperatures of 19–21° C., two containers, one a gallon jar and the other a half-gallon jar, were filled with ice a few hours before testing and, if needed, were refilled immediately before the admission of the flies to the chamber. For temperatures of 37–39° C., a flat pan (30 × 15 × 5 cm.) of hot water, about 50° C., was placed in the chamber to increase the humidity sufficiently to keep the flies from cooling themselves, thus keeping their internal temperature the same as that of the surrounding environment. An infra-red bulb was used as the source of heat and in a matter of minutes the temperature was at 38° C. The ventilator and fan aided in maintaining an even temperature.

After the temperature of the box was set, the flies were allowed to take all the water they wanted in order to prevent a response to the water in the sucrose solution rather than to the sucrose itself. This was administered outside the chamber. Five mounted flies were tested at a time. The flies were allowed to remain in the chamber for 5–10 minutes before testing to reach the temperature of the chamber and were then tested, one at a time, first with water to make certain they had all they wanted, and then from the lowest molarity of sucrose to the highest. A rest period of one or two minutes was allowed after each few tests, so that the fly would not become fatigued to the sucrose.

Each test was made by touching the fly's tarsi to the solution in the Syracuse watch-glass. If the fly dropped its proboscis, it was considered a positive response. If the fly did not drop the proboscis at a low molarity, the fly was tested at successively higher molarities until a positive response was obtained. The flies were always tested from the low molarities to the higher molarities, and the tarsi were rinsed after each few trials to avoid any collection of sucrose on the feet. After the five flies were tested in this manner, they were removed for feeding and watering, and a new batch of five water-sated flies was admitted to the chamber.

Results

The flesh fly, *Sarcophaga bullata,* is easy to handle, because of its convenient size and relative docility. Flies mounted at 24–48 hours of age did not live as long on the mounting rods as those mounted at 96–120 hours. If the animals did not have an opportunity to get all the water they wanted before being anesthetized with ether, they died before or shortly after coming out of the anesthesia, as noted by Frings (1941). Flies that were water-sated before etherization were seemingly unaffected.

Temperatures a few degrees above 40° C. or below 15° C. affected the activity of the flies. The movements of the legs and the proboscis responses were slower than at temperatures of 37–39° C. and 19–21° C. The flies were most active at room temperature.

Approximately 2000 flies were used in this experiment, but over half of these were used to develop experimental techniques. Tests were made on 748 flies, 313 males and 435 females. These flies were subjected to 8241 tests at the three temperature levels.

The median thresholds, as determined by probit analysis (Dethier and Chadwick, 1950; Finney, 1952), were: at 19–21° C., 0.14 *M*; at 27–29° C., 0.06 *M*; at 37–39° C., 0.17 *M*. Significant differences between the per cent response of the flies at the various molarities were determined at the upper probability limits of 0.005 from the tables in Mainland (1948). The lower temperature compared with room temperature (27–29° C.) showed significant differences at 0.6 *M*, 0.4 *M*, 0.2 *M*, 0.1 *M*, 0.08 *M*, 0.06 *M*, 0.04 *M*, and 0.02 *M*. The higher temperature compared with the room temperature showed significant differences at 0.8 *M*, 0.6 *M*, 0.4 *M*, 0.2 *M*, 0.1 *M*, 0.08 *M*, 0.06 *M*, 0.04 *M*, and 0.02 *M* levels. No significant differences between the thresholds of the sexes were noted.

Interpretations and Conclusions

The median tarsal threshold for sucrose (0.06 *M*) for *Sarcophaga bullata* at 27–29° C. is relatively high when compared with that of some other Diptera. For instance, Hassett, Dethier and Fans (1950) reported a median threshold of 0.0098 *M* for the blow fly, *Phormia regina,* and Deonier and Richardson (1935) reported a median threshold of 0.025 *M* for the house fly, *Musca domestica.* Frings and O'Neal (1946), however, found the median sucrose threshold for *Tabanus* to be 0.06 *M* which is the same as that of the flesh fly. A possible explanation for the difference between the median thresholds of *Sarcophaga* and *Tabanus* and those of other Diptera might be that the food habits and habitats of these flies are different from those of the other species.

As to the question of absorption or adsorption in tarsal stimulation, no definite

stand can be taken. From the results, it would seem that penetration was the factor involved at temperatures below room temperature, while at the higher temperatures adsorption was the factor. It is highly improbable, however, that different factors are involved at different temperatures. Perhaps the action, if any, of temperature on the peripheral threshold is masked by the action of temperature on central nervous functions, and thus on general behavior. Possibly neither adsorption *per se* nor penetration is involved, but instead discharge of surface potentials on the sensory hairs. This reaction would be basically independent of temperature. Such a phenomenon would show Hofmeister seriation of inorganic ions and seriations of organic compounds. In such a case, the effects of temperature would be entirely on other systems and thus would give no information of significance on the point in question.

Summary

1. The effects of temperature on the sucrose thresholds of the tarsal chemoreceptors of the flesh fly were studied. Flies were mounted on rods and the thresholds determined in a constant temperature chamber at 19–21° C., 27–29° C. and 37–39° C.

2. A total of 748 flies (313 males and 435 females) were subjected to 8241 tests.

3. The median threshold at 19–21° C. was 0.14 *M*, at 27–29° C. was 0.06 *M*, and at 37–39° C. was 0.17 *M*.

4. On the basis of these data, no definite stand can be taken on the limiting mechanism of tarsal chemoreception.

LITERATURE CITED

Deonier, C. C., and C. H. Richardson, 1935. The tarsal chemoreceptor response of the house fly, *Musca domestica* L., to sucrose and levulose. *Ann. Ent. Soc. Amer.*, **28**: 467–474.

Dethier, V. G., 1951. The limiting mechanism in tarsal chemoreception in blow flies. *J. Gen. Physiol.*, **35**: 55–65.

Dethier, V. G., and L. E. Chadwick, 1948. Chemoreception in insects. *Physiol. Rev.*, **28**: 220–254.

Dethier, V. G., and L. E. Chadwick, 1950. An analysis of the relationship between solubility and stimulating effect in tarsal chemoreception. *J. Gen. Physiol.*, **33**: 589–599.

Finney, D. J., 1952. Probit analysis. A statistical treatment of the sigmoid curve. Second Ed., Cambridge University Press.

Frings, H., 1941. The loci of olfactory end organs in the blow fly, *Cynomia cadaverina* Desvoidy. *J. Exp. Zool.*, **88**: 65–93.

Frings, H., 1947. The mounting of living insects for observation and study. *Turtox News*, **24**: 150–154.

Frings, H., and M. Frings, 1949. The loci of contact chemoreceptors in insects. A review with new evidence. *Amer. Midland Natur.*, **41**: 602–658.

Frings, H., and M. Frings, 1952. Dog biscuit as a larval medium for *Sarcophaga bullata*. *J. Econ. Ent.*, **46**: 183.

Frings, H., and B. R. O'Neal, 1946. The loci and thresholds of contact chemoreceptors in females of the horse fly, *Tabanus sulcifrons* Macq. *J. Exp. Zool.*, **103**: 61–79.

Hassett, C. C., V. G. Dethier and J. Fans, 1950. A comparison of nutritive values and taste thresholds of carbohydrates for the blow fly. *Biol. Bull.*, **99**: 446–453.

Knipe, F., and H. Frings, 1952. An improved type of cage for adult flies. *J. Econ. Ent.*, **45**: 1099.

Mainland, D., 1948. Statistical methods in medical research. I. Qualitative statistics (Enumeration data). *Canad. J. Res.*, **26**: 1–166.

ALTERATIONS IN THE PROTEINS OF SEA URCHIN EGG HOMOGENATES TREATED WITH CALCIUM [1]

PAUL R. GROSS [2]

Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania, and Marine Biological Laboratory, Woods Hole, Massachusetts

The complex of processes involved in the occurrence of a precipitation reaction at the surface of exposed cytoplasm was named the "surface precipitation reaction" by Heilbrunn (1927). A survey of the properties of this reaction in a variety of forms is given by Costello (1932), and a related type of process in muscle fibers is described by Woodward (1948). The most recent treatment of the reaction (henceforth abbreviated SPR), including discussion of its physiological significance, is given in Heilbrunn's (1952) textbook.

The egg of the sea urchin *Arbacia punctulata* provides excellent material for the observation of the SPR. The egg is crushed in sea water, usually between a glass slide and a coverslip. Ordinarily, the egg breaks at one or more points, and the cytoplasm flows out at the site(s) of rupture. The outward movement of the exovates is halted by formation of a precipitation film, and this is usually accompanied by extensive vacuolization within the exovate mass. The Arbacia egg possesses numerous pigment-containing granules (really vacuoles), and these break down in the exovate. The granule lysis and vacuolization reactions are sometimes observed to travel wave-like across the entire cell.

The calcium ions of the sea water are responsible for the initiation of the SPR. If the calcium ions are removed by precipitation with oxalate or by complexing with citrate, the SPR does not occur, and instead, the cytoplasm of crushed eggs is rapidly and uniformly dispersed throughout the volume of medium available to it. This is true of the SPR in all forms studied, from the protozoan Stentor to the egg of the frog (Heilbrunn, 1952).

Heilbrunn (1952) has proposed a theory which attempts to explain such diverse phenomena as stimulation, narcosis, and cell division on the basis of changes in the state of the protoplasmic colloid. Thus, for example, cell division is always preceded by a gelation reaction, the so-called "mitotic gelation."

This physiological gelation process appears to be the analogue of the SPR described above. Indeed, Heilbrunn has proposed that the mitotic gelation is an "internal SPR," differing from the true SPR only in a lesser extent of reaction. Many of the events which accompany the viscosity increase of the cytoplasm in

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[2] National Science Foundation predoctoral research fellow, 1953–54. Present address: Department of Biology, New York University, University College of Arts and Science, University Heights, New York 53, N. Y.

living cells do, in fact, have their striking counterparts in the events of the SPR (Heilbrunn, 1928).

Hultin (1950a, 1950b), impressed by the possible important role of calcium in fertilization and in stimulation in general, studied the effects of the addition of Ca^{++} to homogenates of sea urchin eggs. The innovation of his method was the use of unfertilized eggs which had been freed, prior to their homogenization, of practically all of their ionic Ca. When Hultin added Ca in small quantities to such homogenates, several events were observed: a burst of oxygen uptake in great excess of uptake in the controls, the formation of acid, a sharp increase in the viscosity of the homogenate, and the breakdown of certain cytoplasmic inclusions. These events bear a suggestive resemblance to those which take place at fertilization (Runnström, 1951).

The experiment just described is, however, also a model of the SPR. Indeed, Hultin's preparation *is*, in a sense, the SPR, with the modifying condition that the rupture of the cells is temporally separated from the influx of calcium ions. Certainly, if the events enumerated above suggest those at fertilization, they duplicate the events of the SPR with even greater fidelity.

The experiments of Hultin therefore take on great interest in relation to all phenomena in which the "internal SPR" of cytoplasmic "gelation" are supposed to play a role. Now the SPR obviously involves the formation of an insoluble precipitation membrane (Heilbrunn, 1928), and is likely to be causally connected with a drop in the solubility of some cytoplasmic proteins. The formation of a fibrous gel, such as Hultin (1950b) and Gross (1952) describe would be difficult to account for in another way. Hultin was, however, unable to demonstrate any protein solubility changes as a result of addition of Ca.

It is obviously important to discover whether alterations in the solubility of cytoplasmic proteins do or do not in fact occur. Further, if such alterations can be demonstrated, then it is important that the reaction or reactions leading to this effect be analyzed at the molecular level. Such an analysis would serve to throw light upon the as yet obscure mechanism of the SPR and, perhaps, by extension, upon the processes of gelation and solation which are universal manifestations of vital activity at the cellular level.

This report describes experiments whose purposes relate to this analysis.

I wish to express my sincere gratitude to Dr. L. V. Heilbrunn for his assistance and encouragement during the course of this work.

The electron micrographs were made at the Electron Microscope Laboratory, Marine Biological Laboratory, by Mr. Delbert E. Philpott.

Materials and Methods

Eggs were obtained from female *Arbacia punctulata*. Samples from the pooled batches were tested for fertilizability and poor collections were discarded. The animals were usually injected in the oral region with 0.1–0.5 ml. of isotonic KCl. This treatment induced shedding of the ripe eggs but appeared not to affect immature females. The eggs were collected from six to ten females and quickly washed twice by centrifugation through filtered sea water in order to remove debris. A wash in sea water at pH 5.0 now served to loosen or dissolve

the jelly coats, and these were carried away in the subsequent washings in Ca-free media. Washing by centrifugation and decantation five to eight times in Ca-Mg-free artificial sea water was followed by two washes in 0.35 *M* sodium citrate.

The eggs were now extremely fragile and were easily homogenized after centrifugal collection. Prior to this step, however, the eggs were cooled to 2° C. or below. The homogenization was carried out in a pre-cooled Potter-Elvejhem type of glass homogenizer. A few strokes of the tight-fitting pestle were sufficient. The process described resulted in homogenates of uniform consistency, with few if any intact cells.

As pointed out by Harris (1943), procedures of this type result in the rupture of a certain small percentage of the pigment vacuoles, although most of these bodies are resistant enough to withstand breakage in the course of homogenization. The homogenate was usually prepared with one part of packed eggs and one to ten parts of homogenization medium, this last being isotonic KCl, KCl with buffer (veronal-acetate-HCl), or Ca-Mg-free artificial sea water. The homogenates were strongly self-buffering at a pH of 6.5–6.9.

The details of the several experimental techniques are given in the appropriate sections of the experimental part of this report.

Experiments and Results

1. *Ionic specificity*

The first problem which arises in connection with the striking changes caused by the addition of Ca to the homogenates is that of ionic specificity. Since Hultin (1950b) was able to mimic several of the Ca effects by means of strongly hypertonic salt solutions, the possibility arises that the effects of Ca might be attributed simply to the increased ionic strength of its "isotonic" solutions as compared with that of isotonic solutions of uni-univalent salts. Thus, an isotonic solution of $CaCl_2$ (0.32 *M*) has an ionic strength of 0.96, given by the relation

$$\mu = 1/2 \sum_i c_i z_i^2$$

where μ is called the ionic strength, and the computation consists of summing the products, for each ionic species, of c, the concentration in moles, and z^2, the ionic charge [squared], and dividing the sum over all species by two. By comparison, an isotonic solution of KCl (0.48 *M*) has an ionic strength of 0.48. Since ionic strength, and not molarity, is the value of interest in kinetic salt effects, the above possibility must be investigated.

An aliquot of a homogenate was treated with Ca and a control aliquot was treated with a solution of KCl of identical ionic strength. Thus, 0.1 ml. of 0.32 *M* $CaCl_2$ was added to ten ml. of homogenate and 0.1 ml. of 0.96 *M* KCl was added to the control sample (10 ml.).

The control homogenate retained its normal appearance, whereas the material in the experimental tube underwent, within fifteen minutes, a marked precipitation reaction, as a result of which the viscosity of the homogenate (as a whole) rose. The Ca-treated homogenate formed fiber-like aggregates which adhered to the sides of the test-tube. The granule lysis reaction was evident, and the homogenate

underwent a color change from orange-red to a deep wine-red. As will be shown below, the color change could be prevented by strong additional buffering of the homogenate at pH 7.0.

2. Solubility changes

Hultin (1950b) was unable to observe any alterations in the protein solubility of his homogenates as a result of Ca-treatment. He assumed that such changes do occur, but that the eventual loss of protein from the soluble fraction of the homogenate was balanced and even over-compensated by the release of trichloroacetic acid-insoluble N from the cytoplasmic inclusions which lyse in the presence of Ca.

There is, however, a simple method by which the difficulties caused by granule lysis can be overcome without removing the granules during the reaction period (this latter step constituting a partial fractionation of the homogenate which is not desired at this stage of the investigation). Harris (1943) showed that the pigment granules of the sea urchin egg behave osmotically. Now it is these granules, or vacuoles, which contribute the major quantity of trichloroacetic acid-(henceforth "TCA") insoluble N to the homogenate upon their lysis. Thus, if the granules could be osmotically ruptured in *controls as well* as in Ca-treated homogenates, the phenomenon of granule lysis should have its effect cancelled, and solubility changes in the initially soluble cytoplasmic proteins alone should be measurable.

It is possible to produce osmotic lysis of over 85% of the pigment granules by the addition of water to the homogenate *after* the treatment under investigation. In this way, the effects of Ca^{++} upon the soluble proteins of the homogenate can be studied.

The same basic experimental design was used in all of the solubility experiments. Homogenates were treated with the agent suspected of effecting a solubility change in the proteins. Controls were treated with uni-univalent salt solutions with ionic strengths equal to those obtaining in the experimentals. The tubes containing the homogenates were incubated, either at 2° C. or at 21° C., for various periods. At the end of the incubation period, the homogenates were treated with twice their volume of ice-cold distilled water. This brought about the desired lysis of remaining pigment granules. The homogenates were now diluted with (usually) ten volumes of ice-cold distilled water or *M* KCl, buffered in some cases to pH 7.0. Extraction was carried out in the icebox at 1° C. for one to twenty-four hours. At these low temperatures and relatively great dilutions, the aggregation reactions initiated by Ca were brought to a practical halt.

At the end of the extraction period, the tubes were spun in a Servall angle-head centrifuge at 17,000 to 20,000 g. The clear supernatants were analyzed for their total protein content by one of several methods. The experiments were thus set up to reveal differences in solubility or, more precisely, in sedimentability, the latter being a function of the relative states of aggregation of the soluble or suspended proteins.

For the comparative results required, a quantitative modification of the biuret test, slightly modified from Fine (1935), was found adequate. The results were reproducible, and the slight turbidity which tends to develop at the higher protein

concentrations can often be avoided by suitable dilution and temperature control. Only measurements obtained upon visually non-turbid systems were admitted.

Most of the experiments were repeated with analysis by spectrophotometry. Aside from offering a means of estimating total protein, the absorption spectra of the water extracts provide information concerning the relative distributions of protein and nucleic acid in experimentals and controls.

When the experiment described was performed, there was always a measurable precipitation of protein or protein containing material from the homogenates which had been treated with Ca. At least, at every given relative centrifugal force, more protein was sedimented in the experimentals than was in the controls. When the homogenates were relatively concentrated, that is, when the dilution with homogenization medium was less than five times, the reaction was very rapid at 21° C., although not "instantaneous" in the way that ionic precipitation reactions often are at room temperature.

Depending upon the conditions obtaining, from zero to ninety per cent of the total protein extractable from the whole homogenates by water could be precipitated. Actually, the extraction medium is a very dilute salt solution, since the homogenate itself contains considerable salt.

For reasonable reaction velocities at 21° C., concentrations of Ca^{++} in the range of 0.001 to 0.01 were required. The significance of these levels will be discussed below.

The results suggest that the rate, but not the final extent of solubility loss is dependent upon the concentration of Ca; but this effect could not be noted if dilutions were too great, since the over-all velocity of precipitation is very sensitive to dilution of the homogenate. Indeed, homogenates made with one part of eggs to more than ten parts of medium often failed to give a measureable rate of precipitation in the presence of 0.01 *M* $CaCl_2$, although other Ca effects, such as granule lysis, were observed.

In many experiments where the reaction was permitted to proceed only a short time after treatment with Ca^{++} and a wide range of Ca concentrations was added, the following phenomenon was observed. Plots of solubility loss as a function of Ca^{++} added were often not simple, concave-downward curves, but rather, showed a maximum at about 0.5–1.0 millimols/ liter, followed by a local minimum and finally by steadily increasing values asymptotic with approximately 90% precipitation. The amount of protein lost from the homogenate at the low concentration maxima, when these occurred, was of the order of 20–30% of the total protein extractable with water from the controls.

This is demonstrated by the data in Table I, which shows the results of two typical experiments carried out in successive summers at Woods Hole. Both homogenate preparations were made from 1 : 6 homogenates, *i.e.*, one part of packed eggs homogenized in five parts of isotonic KCl. The considerable variation from one homogenate preparation to another is illustrated by the two sets of data, as are the general characteristics of the relation between solubility loss (or increased sedimentability) and the concentration of added Ca^{++}.

Spectrophotometric methods corroborated the results of the chemical determinations. The water extracts of experimental and control homogenates were centrifuged at 17,000 g for 10 minutes to one hour in the cold, thus brought to water-clarity, and then diluted and read directly in the Beckman spectrophotometer.

TABLE I

Loss in the water solubility of homogenate proteins in the presence of varying amounts of calcium ions. Two separate experiments

Experiment number	Concentration of Ca in mM/liter	Per cent loss in solubility
1A	0.00	0.00
1B	0.57	38.8
1C	5.70	33.4
1D	57.0	72.2
1E	113	81.5
1F	170	94.4
2A	0.00	0.00
2B	1.00	19.0
2C	5.00	14.8
2D	10.0	37.0
2E	100	91.6
2F	500	90.5

Complete absorption spectra in the wave-length region 220 to 300 millimicrons were made for the experimentals and for the controls. The optical density at wave-length 280 millimicrons is a measure of the protein (and aromatic amino acid) content of the sample. The effects of the presence of nucleic acid and of scattering do not introduce a serious error in comparative measurements such as were made here.

In every case, a loss in protein from the Ca-treated homogenate occurred, and was indicated by a reduced optical density at 280 mμ. The absorption spectra had interesting features aside from the changes in density at 280. Figure 1 illustrates the result of a typical experiment. Here, a 1 : 5 homogenate was treated with 0.03 *M* $CaCl_2$ and the control was treated with a solution of KCl of equal ionic strength. After five minutes of incubation at 21° C., each tube was diluted with five volumes of ice-cold distilled water. The extraction was continued in the ice-box for four hours. At the end of this period, both tubes were centrifuged at 17,000 g for ten minutes. The clear supernatants were removed and further diluted with water buffered to pH 8.5 with bicarbonate. Absorption spectra were made in the ultraviolet region.

The upper curve (half circles) in the figure represents the absorption spectrum of the control extract, while the lower curve (solid circles) is that of the experimental. In both cases, the spectrum is that of nucleic acid plus protein, with a peak at 260 millimicrons and an appreciable absorption at 280 millimicrons. Obviously, an over-all loss of water-soluble material has occurred in the experimental system. In addition, the shapes of the curves suggest that the composition of the precipitated material differs slightly from that of the supernatant as a whole, as represented by the upper curve.

The ratio of the molar extinction at 280 mμ to that at 260 mμ has a characteristic magnitude for most proteins and a different, but equally characteristic, magnitude for nucleic acids. Thus, for PNA, this value is in the neighborhood of 0.5, whereas for protein alone it is more likely to have a value near 1.75.[3]

[3] This is not the case for proteins of unusual amino acid composition, such as, for example, protamine.

Warburg and Christian (1942) used these facts as the basis of a method of estimating the relative quantities of protein and nucleic acid in a mixture of the two.

Optical density, the quantity measured in these experiments, is given by the relation

$$D = kcd$$

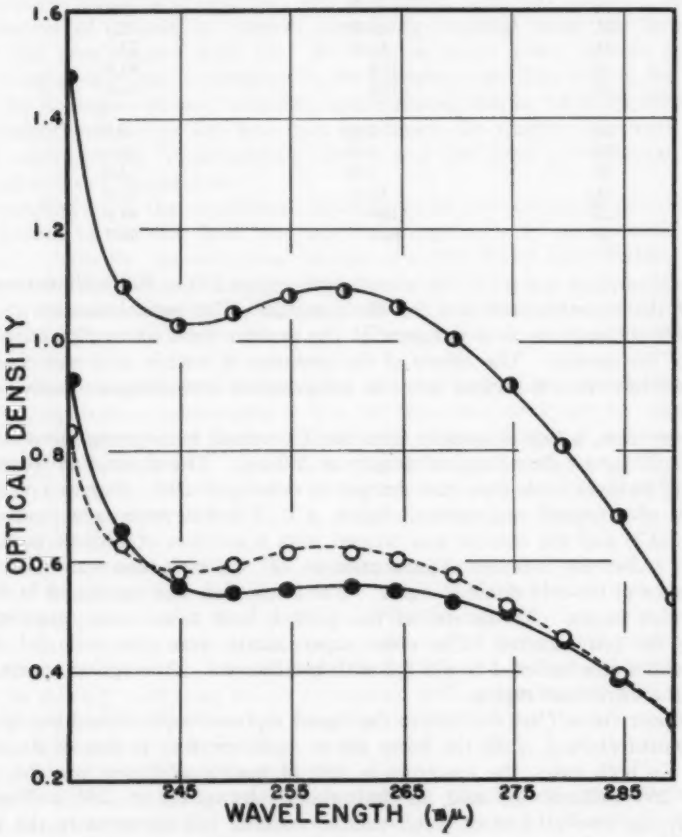

FIGURE 1. Ultraviolet absorption spectra of water extracts of sea urchin egg homogenates. Half circles: control. Full circles: experimental, treated with Ca^{++}. Open circles: calculated curve for experimental. See text.

where D is the optical density, k is a specific extinction coefficient, c is the concentration of absorbing substance, and d is the length of the light path through the sample. The extinction ratio mentioned above is readily obtained from the measured "D" values. For a single sample, the optical density will differ at the two wave-lengths, but the concentration of chromophore and the light path

do not change and are therefore constants, so that

$$\frac{D_{280}}{D_{260}} = \frac{k_{280}}{k_{260}},$$

which is the desired ratio.

The value of this ratio is 0.74 for the upper (control) curve and 0.83 for the experimental curve in Figure 1. This is a significant difference, and indicates that the lower curve represents a chromophore which is relatively poorer in nucleic acid than is the control. But the spectra have been recorded for water extracts, and thus the insoluble, aggregated material in the experimental homogenate must be *richer* in nucleic acid than that in the control.

It is possible, by preparations of several dilutions of the control, to obtain mean values of k (independent of concentration) at the several wave-lengths. These values are multiplied by $d(= 0.996$ cm.) and by "c," which is now that fraction of the absorption (at 280 millimicrons) of the control shown by the experimental. This is equivalent to assuming that the differences between the control and the experimental absorption spectra can be accounted for on the basis of the changes indicated by the 280 mμ absorptions. The product $(k \cdot c \cdot d)$ gives a series of theoretical values for D, and these values are plotted for purposes of comparison with the experimental absorption spectrum. Such a calculated curve is shown by the open circles in Figure 1.

Although, due to the large absorption by nucleic acids even at 280 millimicrons, this type of manipulation can give no quantitative data on the distribution of nucleic acid and protein in the system, it does qualitatively indicate that the experimental water extract is relatively poorer in a component absorbing strongly at 260 mμ than the control extract.

Finally, a preliminary localization of the reaction was attempted in the following way:

A homogenate was centrifuged in the cold at 2000 g for 20 minutes. This deposited a layer of red granules, and no red pigment was left in the supernatant fraction. Treatment of these supernatants was the same as that for whole homogenates.

In every case, the addition of calcium to this supernatant layer brings about a solubility loss on the part of protein components. Figure 2 gives the spectrophotometric analysis of one such experiment, "c" represents the water extract of the control, and "x" that of the Ca-treated supernatant.

Plainly, the material which becomes insoluble in the whole homogenate comes, at least in part, from the fraction devoid of the largest granules.

3. Acid formation

The appearance of acid upon fertilization of the egg of *Paracentrotus lividus* was first reported by Runnström (1933), and this was confirmed for *Psammechinus miliaris* by Borei (1933) and by Laser and Rothschild (1939). These observations led Hultin (1950b) to test for the appearance of acid upon the addition of Ca to his homogenates. Hultin was successful in demonstrating this effect.

The acid formation has considerable significance for the SPR, and accordingly an attempt was made to measure it in our own material.

A homogenate was prepared in the usual way, and then frozen in dry-ice and acetone and dried *in vacuo*. Later, this material was reconstituted with ice-cold distilled water. This material was self-buffered at pH 6.55, as measured with the glass electrode. When the homogenate was brought to 21° C. and treated with $CaCl_2$ (final concentration = 0.005 *M*) the material changed color from brown to deep red within a few minutes. The pH of the system fell from 6.55 to 5.50

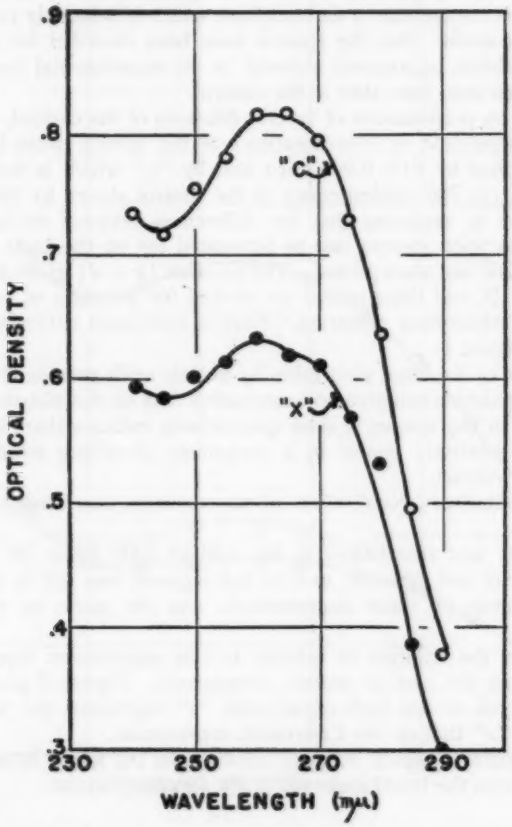

FIGURE 2. Ultraviolet absorption spectra of water extracts of homogenates free of pigment granules. "c" = control. "x" = Ca^{++} — treated experimental.

within 90 seconds. It is easily calculated that, even with buffering neglected, this large release of H^+ cannot be accounted for on the basis of the slightly acid pH of the added $CaCl_2$.

The data indicate that even in the presence of the considerable buffer capacity of the homogenate proteins, etc., the hydrogen ion concentration increased more

than ten times within the first 1.5 minutes after the addition of Ca. The acid formation must thus be of considerable magnitude. In the light of these observations, it is important to ask whether the change in pH is not the factor responsible for the precipitation of protein by Ca.

That this is not the case is easily demonstrated in experiments in which the homogenates are strongly buffered at pH 7. In these systems, the color change does not take place and, of course, the pH remains close to 7, but the loss of protein solubility under the influence of Ca is unaffected.

The matter of color change is of interest, because in the SPR as observed in crushed eggs, the breakdown of the pigment granules is accompanied by a pronounced reddening of the protoplasm of the exovate and often of the whole cell, as the wave of granule lysis sweeps across it. This change in the pigment (echinochrome) released from the vacuoles is particularly striking when eggs are crushed in media somewhat richer in Ca than is sea water. This effect is undoubtedly due to the change in pH of the regions of the cell effected by the SPR.

Two homogenates were prepared and treated in the usual way, except that one was strongly buffered at pH 7.15 with tris-(hydroxymethyl)-aminomethane while the other was unbuffered except by its own ampholytes. Both were treated with the same quantity of Ca^{++} (0.005 *M*) and incubated for a few minutes. The precipitation reaction took place to approximately the same extent in each. Both tubes were centrifuged to sediment the solids, leaving faintly turbid supernatants. These were diluted somewhat and their pH values measured once again with the glass electrode. Absorption spectra of the two preparations were made in the visible region of the spectrum.

The results of the spectrophotometric measurements are plotted in Figure 3. Clearly, the system whose pH was 6.5 was red, while the one with pH = 7.15 was yellow-orange. This shift in absorption is, as closely as it is possible to judge, the same type as that which occurs when the SPR is observed in cells. These color changes are, of course, due to the presence of the pigment echinochrome, which is a pH indicator, apparently even when in combination with its protein ligand in the homogenate or in the egg.

4. Release of non-protein nitrogen

Woodward (1949) reported experiments in which various fractions of Arbacia egg granules were assayed for proteolytic activity. He found low but significant proteolytic activity, increasing from 35 to 50 per cent upon addition of cyanide, in the "granular" fraction of the eggs. Ca-activation of the protease(s) was not demonstrated.

Gross (1952) reported in a note that the whole homogenate of Arbacia eggs contained a Ca-activated protease, with a pH optimum near 6.6. Lundblad (1952), in the course of his extensive investigations upon the proteolytic activity of sea urchin gametes, reported that the weak proteolytic activity of extracts and homogenates of unfertilized eggs was increased by Ca.

Because of the possibility of a relation between proteolysis and coagulation, and perhaps precipitation, of proteins in the homogenates, it seemed worthwhile to attempt a further step in the analysis. Lundblad's assays of protease activity were made by a viscosimetric method. In the light of many investigations such

as those of Örström (1941), demonstrating changes in the non-protein nitrogen (NPN) of eggs after fertilization, it seemed further indicated that one should determine whether NPN increased directly after the addition of Ca to the homogenates. The method used by Lorand (1952) in his studies on fibrinogen was adapted for use in the homogenate system.

Homogenates were prepared in the usual way. Calcium was added in amounts sufficient to give a reasonable rate of precipitation. At intervals, one of the several

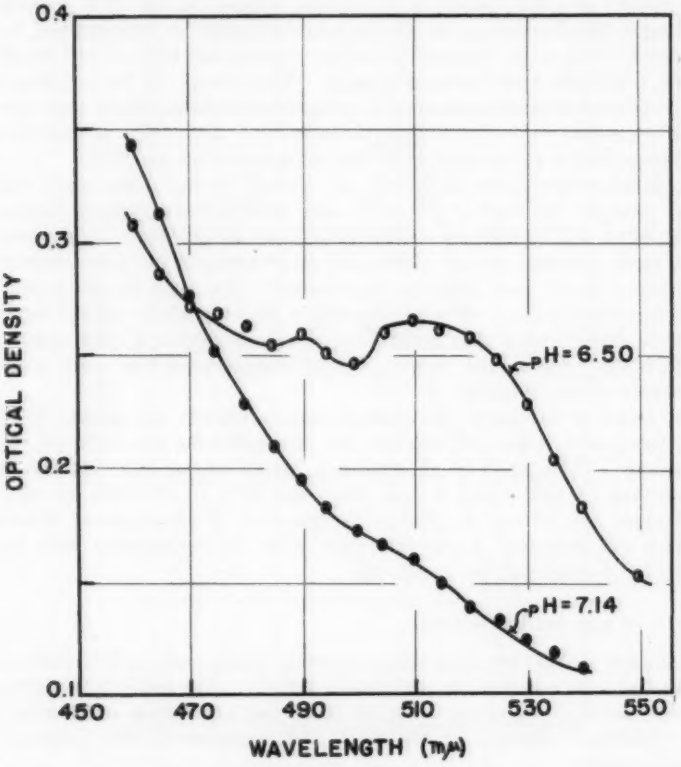

FIGURE 3. Visible light absorption spectra of Ca^{++} — treated homogenate extracts. Half circles: unbuffered. pH = 6.50. Full circles: buffered at pH 7.14.

tubes employed in the experiment was treated with two to five volumes of cold distilled water. An equal volume of 2% monochloroacetic acid was added. After several minutes of gentle shaking, the proteins were precipitated with trichloroacetic acid in a final concentration of 5%. The precipitation was permitted to proceed for 24 hours in the icebox. Finally, the flocculent precipitate was sharply centrifuged down and the clear supernatants collected. The NPN of these super-

natants was determined after digestion in boiling H_2SO_4 and "Superoxol" by direct nesslerization.

Small and very variable increases in NPN were observed to follow the addition of Ca to the homogenates. When measurements were made within the first hour after Ca treatment, the increments were usually of the order of 2–3 per cent, or barely within the limits of precision of the method. With increasing time, however, more NPN appeared. The controls showed little or no protease activity in the absence of Ca when NPN release was the criterion of activity. When the experiments were continued for several hours, however, the controls also showed increments in NPN at the end of the experiment (as compared with a primary control determination at zero time). These increments were always smaller than those observed in the homogenates which contained Ca.

Thus, for example, in an experiment which ran for six hours, the experimental homogenate, which was treated with 0.01 *M* $CaCl_2$, contained 17.3 mg.% NPN (the value for the final diluted assay sample). The control, run along with the experimental, contained 15.6 mg.%, while the primary control, which had been precipitated with TCA six hours previously, contained 14.6 mg.%. These values are the averages of triplicate determinations.

A typical result for an incubation period of 24 hours (at 1° C.) is the following:

Material: Ca-free homogenate, lyophilized and reconstituted.
Treatment: Experimental—0.02 *M* Ca^{++}
Control—KCl of ionic strength equal to that of added Ca solution.
Result: Control: 31.0 micrograms of NPN/ml.
Experimental: 52.2 micrograms of NPN/ml.

5. Electrophoretic experiments

Although electrophoretic examinations of sea urchin egg proteins and protein fractions have been reported previously (Monroy, 1950; Monroy and Monroy Oddo, 1951), earlier work dealt with frozen and dried material. Since the publication of the critical experiments of Lindvall and Carsjö (1951), it has become important to re-examine these results in the light of possible artifacts produced by the lyophilization of fertilized and unfertilized eggs which retain their internal as well as external calcium.

Electrophoresis of extracts of homogenates such as are used in the present work promises to be a useful tool in the further analysis of the SPR-type reactions. The method employed in the experiments was as follows:

Two aliquots of homogenate were obtained. One was calcified, and the other, the control, was treated in the usual way with KCl. After a few minutes incubation, there was the usual treatment with distilled water, followed by extraction in the cold with distilled water. The extracts were finally centrifuged for twenty minutes at 17,000 to 20,000 g. Only clear or very faintly turbid supernatants were accepted for electrophoretic analysis. These were placed in 1-cm. diameter dialysis tubing and dialyzed in the icebox (1° C.) for 48 hours against several changes of 500 ml. of 0.1 N $NaHCO_3$ at pH 8.5 (21° C.). The thoroughly dialyzed colloids were then submitted to electrophoresis in the Perkin-Elmer Tiselius-type instrument, using the 2-ml. cell. The cylindrical lens system was

used for the photographic records of the runs. Photographs were made at definite intervals with the current temporarily turned off. For analysis of these data, the negatives were placed in a photographic enlarger and projected, and the enlarged patterns were traced on cross-section paper. The pattern shown in Figure 4 was prepared in this way, and then inked for reproduction.

The choice of bicarbonate buffer calls, perhaps, for justification. This system was used by Monroy and Monroy Oddo (1951) in their recent experiments upon the protein fractions of fertilized and unfertilized sea urchin material. Their patterns seemed in general to show satisfactory separation. To facilitate com-

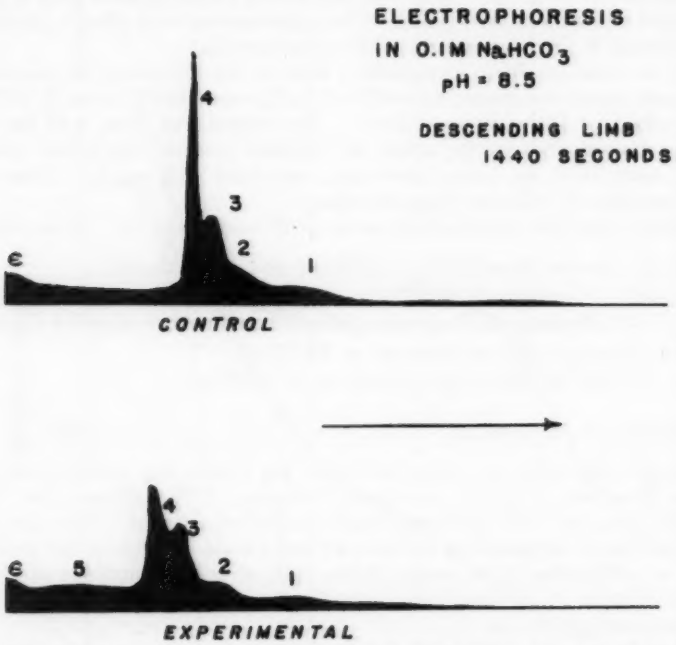

FIGURE 4. Electrophoresis of water extracts of homogenates. Control, untreated. Experimenal, treatment with Ca^{++}.

parison, bicarbonate was used in the present experiments. The separation (resolution) was not as good as could be desired, although sufficient for the comparative data needed. Migration was quite rapid.

Figure 4 shows a typical result. The pattern marked "control" is that of the water extract of a control homogenate. The "experimental" pattern was given by the water extract of a Ca-treated homogenate. Both patterns were photographed at the same interval relative to the commencement of the run. The descending limbs are shown. The field strength (potential gradient) was 6.5 volts/cm. It is apparent that the migration was rapid, and that acceptable resolution was achieved after only 1440 seconds.

In general, three boundaries were observed in all experiments (marked "2," "3," and "4"), with a suggestion of a fourth ("1") in some. Better resolution of these boundaries has already been achieved in experiments with buffers at a lower pH (to be reported elsewhere). In two experiments, a suggestion of a very low, rapidly spreading boundary (marked "5" in Fig. 4) was given by the experimental extracts. In all of the experiments, all boundaries other than the slowest and largest (marked "4") seemed unaffected by the precipitation reaction which had taken place in the experimental. The loss in area of boundary "4," as illustrated in the experiment described, may possibly be ascribed to a selective interaction of the component represented by the boundary with calcium. It remains possible that losses occurred in the smaller boundaries and are not detectable. Nevertheless, the major effect of the calcium ions seems to be upon the largest component.

6. Electron microscopy

If the precipitation reaction under investigation does, indeed, involve one or more specific fractions of the cell's macromolecular or particulate population, then this should be demonstrable by direct visualization of the insoluble products of the reaction. The electron microscope makes such visualization theoretically possible.

Dilute homogenates were prepared. Ca was added to the experimentals, and the controls were treated with KCl of equal ionic strength. Small drops from the experimental and control tubes were immediately withdrawn and placed on collodion films deposited on copper grids.

When the "SPR" in the experimentals had proceeded for the proper interval of time, the reactions were stopped by flooding the mounts with distilled water and removing the large drops formed with filter paper. This process was repeated several times, with increasing intervals between flooding and withdrawal of the distilled water. This process served to 1) dissolve away the salts, 2) lyse the large pigment granules and wash away their ghosts, and 3) remove all water-soluble parts of the preparation. In addition, the mechanical disturbance occasioned by the withdrawal of the drops removed all but the most tenaciously sticky materials from the collodion.

The samples were either permitted to dry immediately, or they were first fixed with 0.2% phosphotungstic acid for two minutes and then washed again and dried. Finally, all preparations were shadow-cast with chromium. The electron micrographs were made with an RCA model EMU microscope.

Fixed and unfixed preparations gave essentially the same results, except that the materials fixed with phosphotungstic acid showed particles which were slightly more discrete and less flattened. Some slight syneresis was observed in these preparations. The discreteness and lesser degree of flattening are probably due to a certain rigidity imparted to the proteins by the fixative action of the acid, rather than by its electron stain properties. The photographs presented in this report were all made from fixed preparations.

Figure 5 is typical of the results obtained with the control preparations. Whether fixed or unfixed, these preparations showed only a thin layer of material blanketing the collodion substrate. The washing procedure appears, in these preparations, to have removed not only the water-soluble materials, but in addition

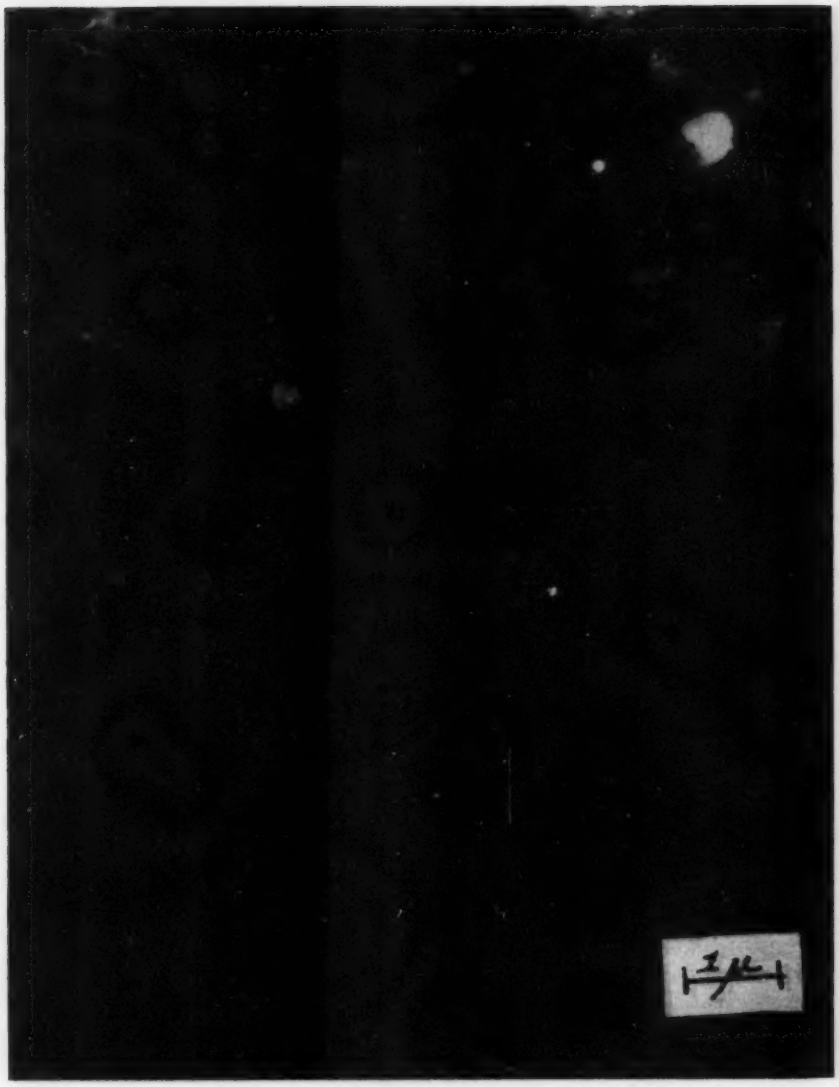

FIGURE 5. Electron micrograph of control preparation. See text. Magnification: 15,000×. Collodion surface with some low-molecular materials.

most of the water-insoluble materials. This is a result of the mechanical disturbance effected by the repeated withdrawal of the water droplets. Such a lack of larger structures in the control preparations is indeed fortunate, for such materials as do appear as a rule in the experimental preparations and are absent from the controls must in some way have had their solubility in water or their adherence to collodion sharply altered by the treatment with Ca. The specimen in Figure 5 is essentially clean collodion with perhaps some deposit of relatively low-molecular material. The magnification is 15,000 diameters.

Figure 6 is a typical experimental specimen, seen at a magnification of 28,000 diameters. Plainly, the result of the Ca-treatment has been the formation of a flocculent precipitate, insoluble in water, and involving particles other than the large pigment granules, which have been lysed and washed away by the preparation method. At the magnification shown, it is not possible to resolve the fine-structure of the microflocs to any appreciable extent; however, the edges of the floc shown, as well as the very small aggregates on the collodion, suggest that the basic aggregating unit is small and approximately spherical. Although there is a suggestion of linear aggregation on the figure, there is in general no pronounced order in the flocs as observed. They appear always, at this magnification, to be of the close-packed, polyfunctional type.

When observed at the highest magnifications, such as that in Figure 7 (110,000 ×), the thinnest microflocs reveal their fine-structure. The "linear" aggregates seen only poorly at the lower magnifications dominate these pictures as long, ropy strands of material, made up of (highly flattened) spheres in linear array. Since high surface pressures are exerted upon the specimens with the air-drying technique used in these preparations, it is likely that the discs were originally spheres. The aggregation reaction may thus consist in part of a difunctional type of polymerization at the earlier stages and then an irregular and extensive interchain packing superimposed.

It is probable that the extreme irregularity of the final packing in the microflocs observed is a result of 1) the drastic treatment of the material during the preparation of the specimens, and 2) syneresis due to the fixative. Of course, since the particles in question do not stick (probably because they are not aggregated) in the control, the foregoing considerations do not apply to controls.

Several representative plates at the high magnification (electronic magnification = 13,250 ×) were subjected to microscopic examination and measurement of the images of the small particles making up the aggregates. These particles, which it should be remembered do not remain on the control mounts, are distributed in diameter about a mean value of 380 Å. The distribution is skewed with the mode displaced somewhat toward the smaller diameters, and the variance is rather large (S.D. = 80 Å.).

The large variance, although not unusual in electron microscopic preparations of cell fractions, indicates, probably, rather extensive deformation of the original particles.

Discussion

The objectives of these investigations were two-fold: first, the clarification of the question of protein solubility changes as a result of the "test-tube SPR" and

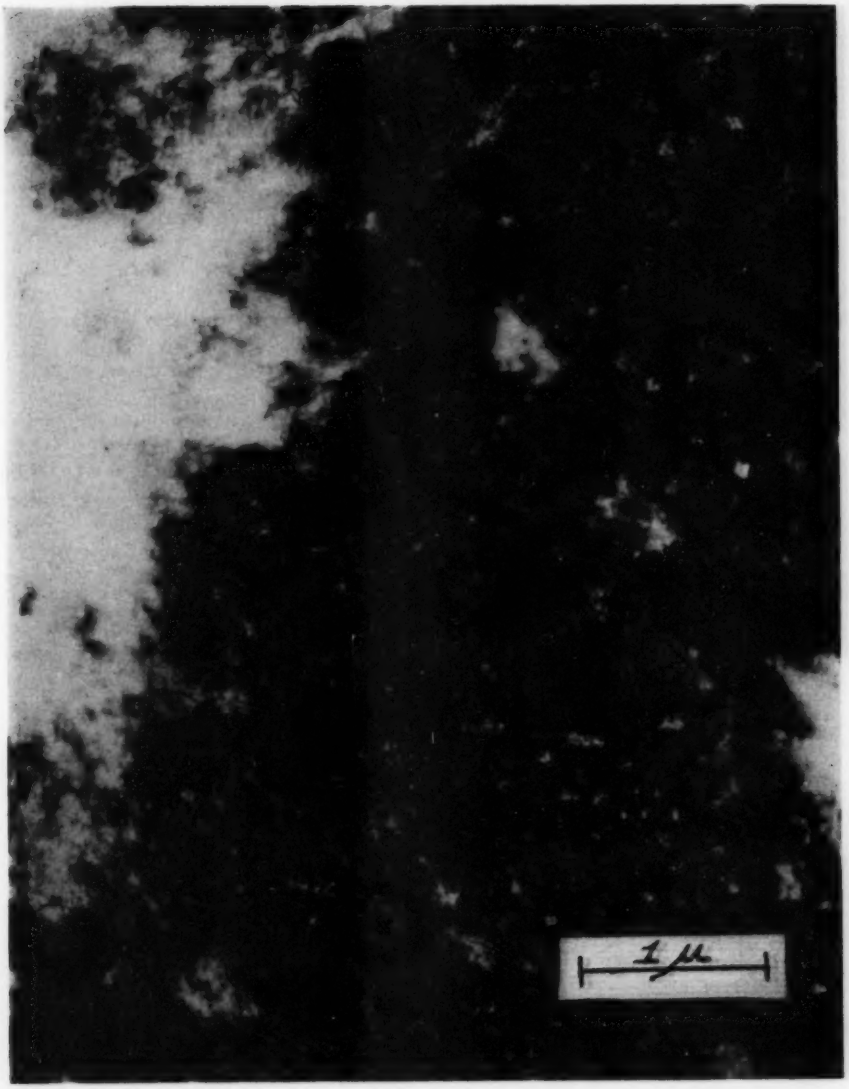

FIGURE 6. Electron micrograph of experimental preparation. See text. Magnification: 28,000 ×.

second, the construction of a reaction mechanism for the over-all phenomenon. By extension, such a mechanism would be of great utility in interpreting the molecular basis of colloidal alterations in the cytoplasm of living cells.

The addition of calcium ions *has* been found to reduce the solubility of some proteinaceous constituent of the homogenate, or at least, the sedimentability of this constituent is increased, which can be interpreted as an increase in aggregation.

But the second objective remains remote. One has had, so far, to be content with the acquisition of a spectrum of *possible* reactions involved in the macroscopic event called the SPR. Thus the experiments reported make no attempt to test interrelations of reactions, but rather to establish that certain reactions do occur as a result of the initiation of a "test-tube SPR" by Ca.

The fact that a local level or optimum for precipitation exists in the homogenate with regard to concentrations of Ca added at the level of 0.001 to 0.01 *M* may be related to the following facts.

1) The "physiological" rates of reaction (with half-time of the order of seconds to minutes) at 21° C. are the ones observed at these concentration levels.

2) The free Ca of unfertilized eggs is of the order of 0.0005 *M* (Mazia, 1937). Also, the concentration below which an SPR (in whole eggs) will *not* take place is very close to this, *viz.*, 0.0003 *M*. When the *free* Ca concentration of the cytoplasm is increased beyond this general level (5×10^{-4} *M*), the sea urchin egg experiences a protoplasmic gelation, or clotting. Mazia (1937) demonstrated that the fertilization of sea urchin eggs results in the liberation of *free* (ultrafilterable) calcium in the egg. Furthermore, the concentration of free Ca in the egg was shown to increase by 0.001 *M* upon fertilization, placing the total free Ca of the fertilized egg at 0.0015 *M*.

It may be significant that this level of Ca concentration, which we may term "physiological" for the system studied, is at one end of the range indicated as required for reasonably rapid rates of reaction in the *in vitro* system. Considering the dilution of reactants which takes place in the preparation of an homogenate, the correspondence of these concentration levels is surprisingly good.

The fact that the precipitated material contains nucleic acid may explain why such large proportions of the total cellular protein can ultimately (at very high Ca concentrations) be aggregated, and still appear to comprise, through most of the concentration range, a single fraction or group of fractions closely related. First, it is safe to assume that most of the nucleic acid being precipitated is PNA. Villee *et al.* (1949) have shown that the DNA of the uncleaved Arbacia egg corresponds to only about 6% of the total nucleic acid, and according to Abrams (1951) this figure is probably too high. The small quantity of DNA present in the homogenate could then hardly account for the large proportion of the total nucleic acid which can be precipitated by small quantities of Ca. Finally, if the affected fraction contains PNA, it may be one of the PNA-rich particle fractions of the cytoplasm, in which case it could conceivably represent a large proportion of the total protein of the egg.

The electrophoretic experiments suggest that the fraction(s) undergoing change under the influence of Ca are a part of one of the largest macromolecular or small-particulate fractions of the cell. The electron microscope pictures show that the particle in question is (after reaction with Ca) a large macromolecule or a very

small complex cytoplasmic particle and that it is probably globular before polymerization.

It is specified that the particle has these characteristics *after* treatment with Ca, because a careful inspection of high magnification photographs, such as that in Figure 7, reveals a fine-structure within the particles themselves, at the level of 40–70 Å. Probably, this represents the fine structure of the particles, but there is no evidence that the 380 Å particle is not *itself* an aggregation product resulting from the addition of Ca. The latter is, however, rather unlikely for many reasons, among which the results of the electrophoretic experiments and the probable large decrease in entropy in such a specific aggregation are prominent.

Thus, the experimental evidence seems to indicate a process in which Ca brings about the aggregation of certain specific particles of the cytoplasm into an insoluble coagulum. The specificity of this reaction, as well as the quality of its insoluble product, fit well with what is known of the SPR and of its normal analogue, the cytoplasmic sol-gel transformation.

The other events of the test-tube SPR also have their analogues in the SPR of the whole cell, and, interestingly, in the events of fertilization and stimulation. Whether the acid formation observed in the system under investigation here is related to that which occurs at fertilization (Runnström, 1933) is, of course, not decided by these experiments. Nevertheless, there is a strong suggestion that this might be the case. The color changes appearing in the test-tube reaction have their direct parallel in the SPR, and suggest that injury can produce large local increases in the hydrogen ion concentration of protoplasm. This is, of course, a fact generally recognized from other types of experiments (*e.g.,* Chambers and Pollock, 1927). The proteolysis measured in the *in vitro* system has its analogue in fertilization (Lundblad, 1950) and, *in vivo,* probably in the form of a release of NPN (Örström, 1941).

It is perhaps significant for the evaluation of the "colloid chemical" theory of stimulation of Heilbrunn (1952) that the *in vitro* system here studied has similarities to the clotting of blood in two heretofore undemonstrated areas. Not only do both reactions involve initiation or activation by Ca ions, but both involve polyfunctional polymerizations of macromolecular monmers, and both involve some step in which a proteolytic enzyme is activated and small quantities of NPN are released. This must not be taken to indicate that the precipitation reaction in the homogenate is necessarily mediated by a proteolytic enzyme. Such an enzyme is indeed present, but its participation in the reaction leading to aggregation in the homogenate must be demonstrated by methods not reported here. Such methods, particularly the use of alternating low and high temperatures, and the addition of clotting inhibitors are currently in use in this laboratory. It is also interesting, in this connection, that the fat solvent anesthetics such as ether and alcohol inhibit the *in vitro* reactions between the homogenates and calcium. These results will be more fully reported in another publication.

It should be noted that the formation of true fibers, such as those of fibrin (Hawn and Porter, 1947), is not observed in the SPR-type reaction. Plainly, the fibrin-clot type of structure could not, and does not occur within a living cell. It is for this reason that it is erroneous to speak of cytoplasmic gelation or of "protoplasmic clotting." The word "gel" defines a system of infinite viscosity,

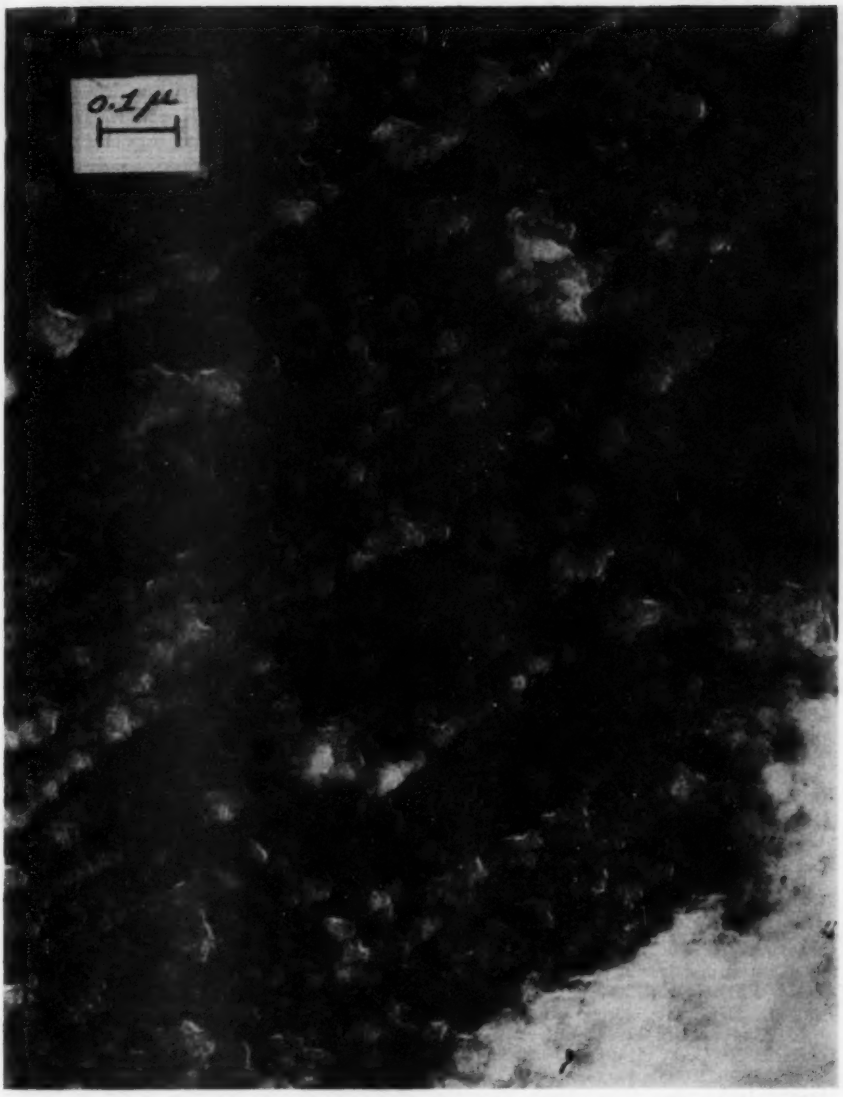

FIGURE 7. Electron micrograph of experimental preparation. See text. Magnification: 110,000 ×.

which is the case with a normal fibrin clot. But in a living cell, the cytoplasmic viscosity increases only by a factor of three to six in the usual sol-gel transformation. One presumes that in the SPR, these changes would increase in magnitude, and could indeed lead to a gel.

From these considerations, one may conclude that an aggregation process that might be expected to occur in a living cell, or even in the SPR, should certainly not lead to the formation of fibers of great length. Rather, the expectation is a reversible aggregation of small units into networks of dimensions easily accommodated within the confines of a single cell. The nature of the aggregation process observed in the homogenate system is thus, at least teleologically, justified.

Summary

1. The addition of Ca^{++} to homogenates of Arbacia eggs which have been previously made calcium-free results in the loss in solubility of certain protein constituents of the homogenate.

2. The precipitated material is rich in nucleic acid, and comes, at least in part, from the fraction non-sedimentable at 2000 g.

3. The addition of Ca^{++} to homogenates activates a proteolytic enzyme and leads to the release of small amounts of additional non-protein nitrogen.

4. None of these reactions can be ascribed to the high ionic strength of solutions of calcium salts as compared with that of isosmotic solutions of uni-univalent salts.

5. The fraction undergoing solubility loss appears to be electrophoretically homogeneous, since it is represented by the largest and slowest-migrating boundary in the water extract of the homogenate.

6. Electron microscope observations reveal that the aggregating material consists of spherical or discoidal particles of (flattened) diameter 380 Å. The aggregation pattern observed is that of microflocs.

7. The possible significance of these observations for the mechanism of the surface precipitation reaction and for sol-gel changes in the cell interior is discussed.

LITERATURE CITED

Abrams, R., 1951. Synthesis of nucleic acid purines in the sea urchin embryo. *Exp. Cell Res.*, **2**: 235–242.

Borei, H., 1933. Beiträge zur Kenntnis der Vorgänge bei der Befruchtung des Echinodermeies. *Zeitschr. Vergl. Physiol.*, **20**: 258–266.

Costello, D. P., 1932. The surface precipitation reaction in marine eggs. *Protoplasma*, **17**: 239–257.

Chambers, R., and H. Pollock, 1927. Micrurgical studies in cell physiology. IV. Colorimetric determinations of the nuclear and cytoplasmic pH in the starfish egg. *J. Gen. Physiol.*, **10**: 739–755.

Fine, J., 1935. The biuret method of estimating albumin and globulin in serum and urine. *Biochem. J.*, **29**: 799–803.

Gross, P. R., 1952. A study of colloidal changes in sea urchin egg homogenates. *Biol. Bull.*, **103**: 293.

Harris, D. L., 1943. The osmotic properties of the cytoplasmic granules of the sea urchin egg. *Biol. Bull.*, **85**: 179–192.

Hawn, C. v Z., and K. R. Porter, 1947. The fine structure of clots from purified bovine fibrinogen and thrombin: a study with the electron microscope. *J. Exp. Med.*, **86**: 285–292.

HEILBRUNN, L. V., 1927. The colloid chemistry of protoplasm. V. A preliminary study of the surface precipitation reaction of living cells. *Arch. f. Exp. Zellforschung*, **4**: 246–263.

HEILBRUNN, L. V., 1928. The colloid chemistry of protoplasm. Protoplasma Monographien Vol. 1. Berlin.

HEILBRUNN, L. V., 1952. An outline of general physiology. W. B. Saunders Co., Third edition. Philadelphia.

HULTIN, T., 1950a. On the oxygen uptake of *Paracentrotus lividus* egg homogenates after the addition of calcium. *Exp. Cell Res.*, **1**: 159–168.

HULTIN, T., 1950b. On the acid formation, breakdown of cytoplasmic inclusions, and increased viscosity in Paracentrotus egg homogenates after the addition of calcium. *Exp. Cell Res.*, **1**: 272–283.

LASER, H., AND LORD ROTHSCHILD, 1939. The metabolism of the eggs of *Psammechinus miliaris* during the fertilization reaction. *Proc. Roy. Soc. Lond., Ser. B*, **126**: 539–556.

LINDVALL, S., AND A. CARSJÖ, 1951. On protein fractions and inorganic ions in sea urchin eggs, fertilized and unfertilized. *Exp. Cell Res.*, **2**: 491–498.

LORAND, L., 1952. Fibrino-peptide. *Biochem. J.*, **52**: 200–203.

LUNDBLAD, G., 1950. Proteolytic activity in sea urchin gametes. I. Activity in untreated extracts. *Exp. Cell Res.*, **1**: 264–271.

LUNDBLAD, G., 1952. Proteolytic activity in sea urchin gametes. II. Activity of extracts and homogenates of the egg subjected to different treatments. *Arkiv för Kemi*, **4**: 537–565.

MAZIA, D., 1937. The release of calcium in Arbacia eggs on fertilization. *J. Cell. Comp. Physiol.*, **10**: 291–304.

MONROY, A., 1950. A preliminary electrophoretic analysis of proteins and protein fractions in sea urchin eggs and their changes on fertilization. *Exp. Cell Res.*, **1**: 92–104.

MONROY, A., AND MONROY ODDO, 1951. Solubility changes of proteins in sea urchin eggs upon fertilization. *J. Gen. Physiol.*, **35**: 245–253.

ÖRSTRÖM, Å., 1941. Über die Stickstoffraktionen im Ei von *Paracentrotus lividus* vor und nach der Entwicklungserregung, und über ihre Bedeutung für den osmotischen Druck und den Stoffwechsel. *Arkiv för Kemi, Mineral. och Geol.*, **15 A**: 1–13.

RUNNSTRÖM, J., 1933. Spaltung und Atmung bei der Entwicklungserregung des Seeigeleies. *Biochem. Zeitschr.*, **258**: 257–279.

RUNNSTRÖM, J., 1951. Problems of fertilization as elucidated by work on sea urchins. *Harvey Lectures*, **46**: 116–152.

VILLEE, C. A., M. LOWENS, M. GORDON, E. LEONARD AND A. RICH, 1949. The incorporation of P^{32} into the nucleoprotein and phosphoprotein of the developing sea urchin embryo. *J. Cell. Comp. Physiol.*, **33**: 93–112.

WARBURG, O., AND W. CHRISTIAN, 1942. Isolierung und Kristalisation des Gärungsferments Enolase. *Biochem. Zeitschr.*, **310**: 384–421.

WOODWARD, A. A., JR., 1948. Protoplasmic clotting in isolated muscle fibers. *J. Cell. Comp. Physiol.*, **31**: 359–394.

WOODWARD, A. A., JR., 1949. Proteolytic enzymes in the cytoplasmic granules of Arbacia eggs. *Biol. Bull.*, **97**: 264.

A TIDAL RHYTHM IN BEHAVIOR OF MELANOPHORES IN AUTOTOMIZED LEGS OF UCA PUGNAX

MARGARET N. HINES [1]

Dept. of Biological Sciences, Northwestern University,[2] *Evanston, Illinois*

Studies of the behavior of marine animals have shown that a number of forms which normally are exposed to periodic tidal variations in their environment possess patterns of activity which are correlated with the tides. In some instances these tidal rhythms have been found to persist under constant conditions in the laboratory. The literature of this subject has been reviewed by Brown, Fingerman, Sandeen and Webb (1953).

The chromatophore system of the fiddler crab, *Uca pugnax,* has provided the basis for considerable study of rhythmic behavior. These crabs possess a diurnal rhythm of color-change, such that they are dark during the day, due to dispersed melanin within the chromatophores, and light at night, due to concentrated melanin. These diurnal variations are supplemented by a tidal rhythm of melanin dispersion possessing 12.4-hour cycles (Brown, Fingerman, Sandeen and Webb, 1953). The simultaneous possession of diurnal and tidal rhythms results in semilunar cycles of 14.8-day frequency, since it is at this interval that a given phase of tide coincides with a given time of day.

The daily pattern of oxygen consumption of *Uca* also shows variations which are tidally and diurnally rhythmic (Brown, Bennett and Webb, 1953). The diurnal rhythm in metabolic rate was demonstrated by determining the average oxygen consumption during each hour of the day of a 15-day period. The tidal rhythm was demonstrated by utilizing data for a 29-day period in such a fashion that the diurnal rhythm was randomized.

Both color-change and oxygen consumption are hormonally regulated processes. However, as yet there has been no direct demonstration of variations in the titer of hormonal substances in the blood of *Uca.* The chromatophores of legs autotomized by the crabs remain, at least initially, in contact with the blood-borne substances which must normally determine the degree of dispersion of the pigments. It was believed that the changes in the state of melanin dispersion following leg autotomy might reflect, in some fashion, the normal variations in hormonal concentrations which occur in the intact animal. The experiments to be reported here were begun in an attempt to determine what factors might be involved in the behavior of melanophores following autotomy.

Materials and Methods

Uca pugnax were collected at Chapoquoit, near Woods Hole, Massachusetts, and maintained in the Marine Biological Laboratory in large white dish pans

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[2] Present address: Department of Biology, College of Wooster, Wooster, Ohio.

containing sea water to a depth of a few millimeters. The animals were maintained in continuous illumination which varied in intensity from 2 ft.c. during the night to approximately 15 ft.c. during the day. The stock group was replenished with freshly collected animals at intervals of two to four days. In no experiment were animals used which had been in the laboratory longer than four days.

In order to determine the behavior of the melanophores in autotomized legs animals were induced to autotomize two or three legs by applying pressure on a distal segment of the leg. The autotomized legs were placed in sea water in Syracuse watch glasses. In determining the degree of melanin dispersion characteristic of any group of legs the average index of the melanophores on the anterior surface of the meropodite of each leg was estimated, using the Hogben and Slome scale, in which 1 describes completely concentrated pigment, 5 describes completely dispersed pigment, and 2, 3, and 4 describe graded intermediate degrees of dispersion. The average of the indices estimated for the several legs of each group was considered to describe the degree of melanin dispersion characteristic of that group.

The experiments were conducted during the summer of 1953. The times of high and low tides referred to in this paper are those occurring on the day of the experiment at the site of collection of the animals.

Experiments and Results

Series I. Characteristics of the behavior of melanophores during the hour following autotomy

The average rate of concentration in melanophores of fifty legs obtained from twenty-five animals selected randomly from the stock group was determined during the hour after the legs were caused to autotomize. The legs were divided into five groups, each consisting of two legs obtained from each of five animals. The average chromatophore index of each group was determined immediately following autotomy (10 A.M.), and again 10, 20, 30, 45, and 60 minutes following autotomy. The average chromatophore index of all legs at the time of autotomy was 4.7; the average values of the subsequent readings were 4.6, 4.3, 3.8, 3.5, and 3.5.

This procedure was repeated at 5 P.M. on the same day. The average chromatophore index of the fifty legs at the time of autotomy was 4.0. The average stages at 10, 20, 30, 45, and 60 minutes following autotomy were 3.7, 3.1, 2.9, 2.5, and 2.7, respectively.

The average chromatophore index determined for each group of ten legs at each reading is plotted in Figure 1. By calculating the slopes of the various intervals of each of these curves, it was determined that the maximum rate of concentration occurred during the first 30 minutes following autotomy in eight of the ten groups. The standard deviation of the 30-minute values obtained for the five groups of legs in the first experiment is $\pm$ 0.49, and that for the values obtained in the second experiment is $\pm$ 0.33.

It was concluded that in frequent determinations of the behavior of the melanophores in autotomized legs, the average chromatophore stage of a group of ten legs determined 30 minutes after autotomy could be used as a good index of the direction and the relative rates, and total amount of change in the melanophores. Numerous additional observations have also indicated that the chromatophore stage observed

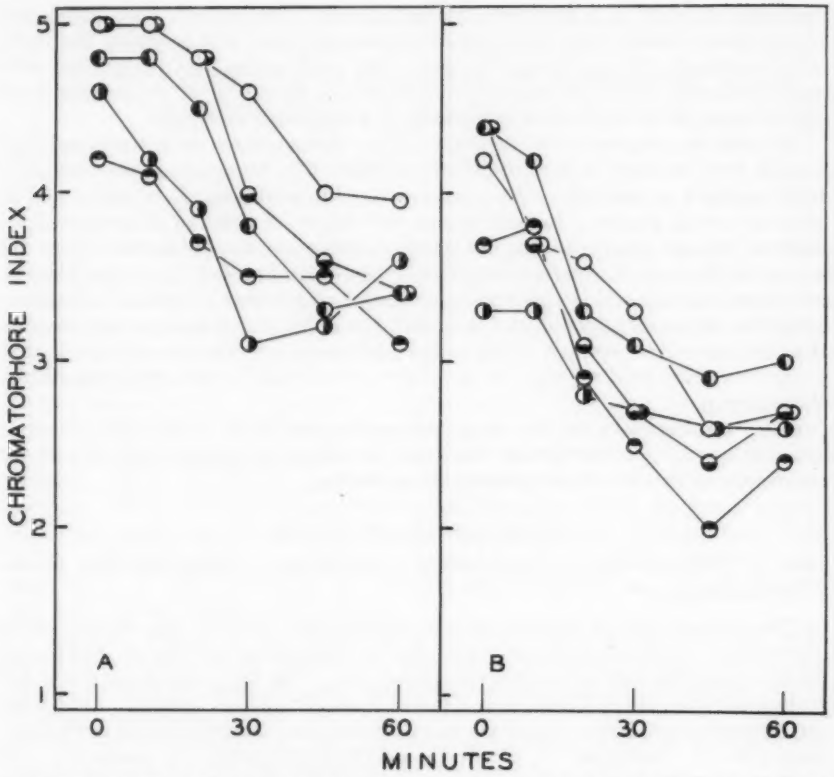


FIGURE 1. The behavior of melanophores of *Uca pugnax* during the hour following autotomy (Series I). The values for the five groups of legs autotomized at 10 A.M. are represented in the A portion of the graph, while those for the legs autotomized at 5 P.M. are given in the B portion.

30 minutes following autotomy is as accurate an index of such melanin changes as one could find.

Series II. Behavior in legs with maximally dispersed melanin

Crabs maintained in an illumination of 100 ft.c. were used in preliminary studies of factors affecting the melanophore behavior in autotomized legs. For each determination two legs were removed from each of six animals in which the melanin was maximally dispersed, and the average chromatophore index of the groups was determined 30 minutes after autotomy. A total of 32 determinations were made between 5 A.M. and 10 P.M. on 7 days.

These data are summarized in Figure 2. The difference between the time of day at which autotomy was induced and the time of day of the nearest high tide was calculated for each determination; the average chromatophore index observed 30

minutes after autotomy has been plotted against the difference so obtained. The lowest 30-minute values, indicative of the greatest concentration, were observed when the determinations were made near the time of high tide. The highest 30-minute values were observed when autotomy was induced between the times of a high tide and a low tide.

Series III. Relationship between the time of high tide and the behavior of melanophores

In order to determine any variation in melanin concentration which might be correlated with the time of day at which high tide occurs, observations were made through the daytime over a period of 23 days. Crabs of the stock group maintained in continuous illumination of 2- to 15-ft.c. intensity were used in this study. Autotomy of two legs of each of five animals, selected randomly, was induced at half-hour intervals. The average melanophore stage of each group of legs was deter-

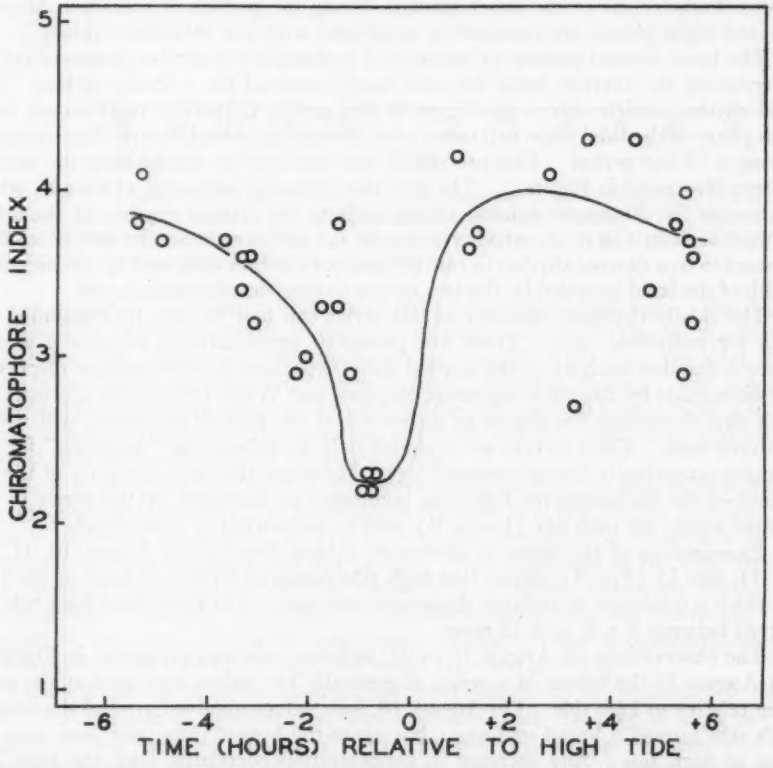


FIGURE 2. The 30-minute values in legs autotomized by animals with maximally dispersed melanophores (Series II). The values are plotted against the difference between the time of day at which autotomy was induced and the time of day of the nearest high tide.

mined at the time of autotomy and again 30 minutes afterwards. During the first few days of the series observations were made between 8 A.M. and 5 P.M. During the latter portion of the series observations were extended to include earlier and later periods.

In analyzing these data the initial melanophore indices of the two groups of legs studied in any one hour were averaged to give a measure of the average dispersion of melanin for the population at that time. Also the 30-minute values obtained for the two groups observed during each hour were averaged to give a measure of the characteristic melanophore stage at the end of the half-hour following autotomy at that time of day. The differences between these average initial values and their respective 30-minute values provide the basis for calculating the rates of concentration characteristic of each hourly period.

The behavior of melanophores of legs obtained from this stock population clearly reflects the diurnal rhythm of color-change of the intact animals. In only a few cases did dispersion of melanin occur following autotomy. Consequently the low values characteristic of the intact animals during the periods of transition between day and night phases are consistently associated with low 30-minute values.

The basic diurnal pattern in behavior of melanophores can be demonstrated by determining the average value for each hourly interval for a 15-day period. The tidal rhythm, which also is significant in this series, is thereby randomized, since each phase of the tidal cycle will occur once in relation to each hourly determination during a 15-day period. This procedure was employed in determining the diurnal pattern illustrated in Figure 3. The behavior following autotomy (Curve B, which represents the 30-minute values) clearly reflects the diurnal rhythm of the intact animals seen in Curve A, which represents the initial values. In addition, there appears to be a diurnal rhythm in rate of concentration, as indicated by the increased width of the band bounded by the two curves during the afternoon hours.

The tidally rhythmic behavior in this series can best be seen by examining the data for individual days. These are presented graphically in Figures 4 and 5. Since a detailed analysis of the normal daily variations in melanophore dispersion has been made by Brown, Fingerman, Sandeen and Webb (1953), the Curve A for each day, describing the degree of dispersion at the time of autotomy, will not be analyzed here. These curves are included only to indicate the "baselines" for the changes occurring in the autotomized legs. However, the daily character of the behavior of the melanophores following autotomy, as described by the series of 30-minute values for each day (Curve B), will be considered in some detail.

Examination of the series of 30-minute values observed on August 10, 11, 12, 13, 14, and 15 (Fig. 4), shows that high tide occurred within an hour of the time at which a minimum of melanin dispersion was seen. On these days high tide occurred between 8 A.M. and 12 noon.

The observations on August 17 to 22, inclusive, are also presented in Figure 4. On August 17 the lowest in a series of generally low values was seen at the usual time relative to high tide. On August 18, four values obtained around the time of high tide formed a broad minimum, but again the lowest value was seen near the time of high tide. Any increase in concentration correlated with the high tide which occurred at 3 P.M. on August 19 was more or less masked by the generally increasing degrees of concentration which occurred during the afternoon.

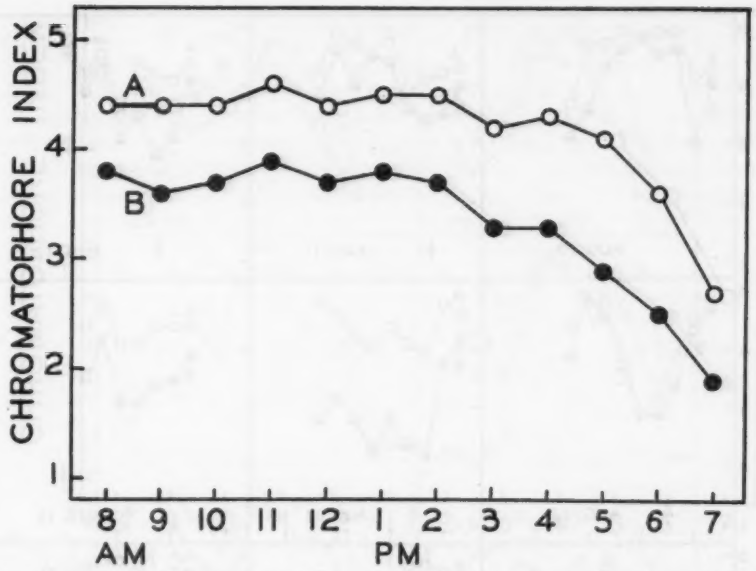
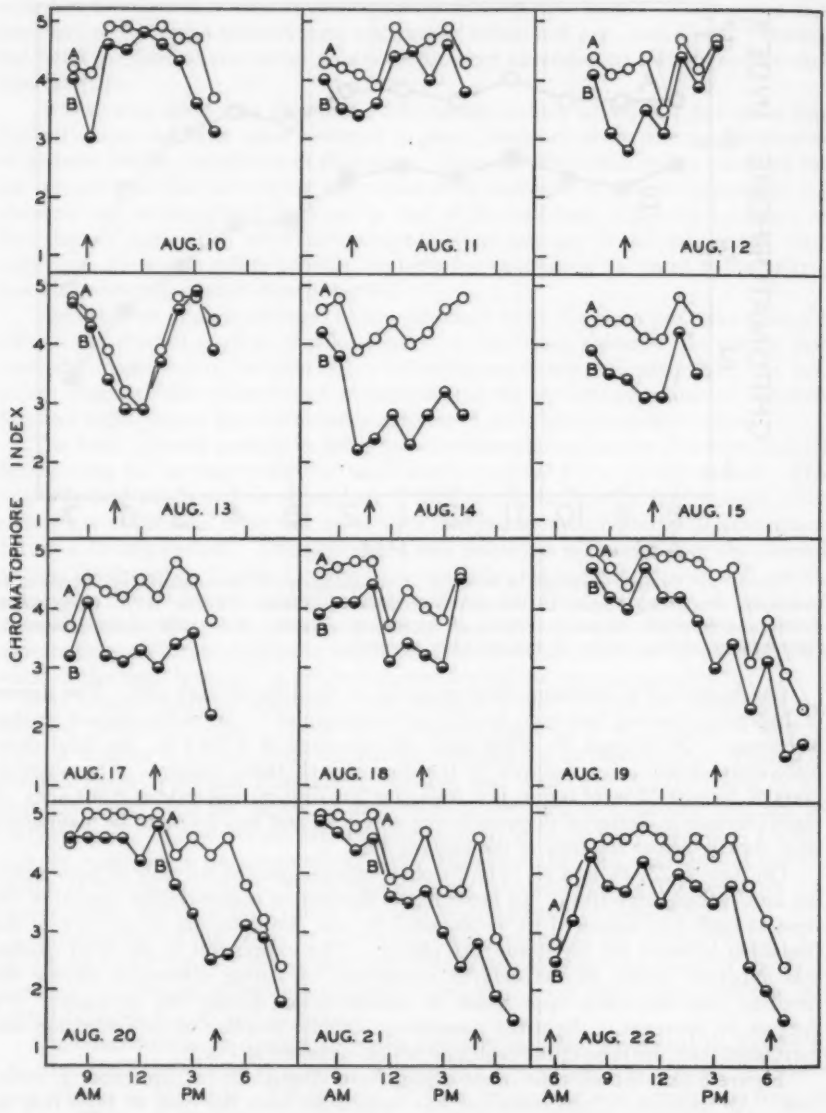


FIGURE 3. Diurnal variation in behavior of melanophores in autotomized legs, obtained by averaging data from August 18 through September 1, inclusive (Series III). Open circles (Curve A) represent the average values at the time of autotomy, while solid circles (Curve B) represent the average values 30 minutes after autotomy.

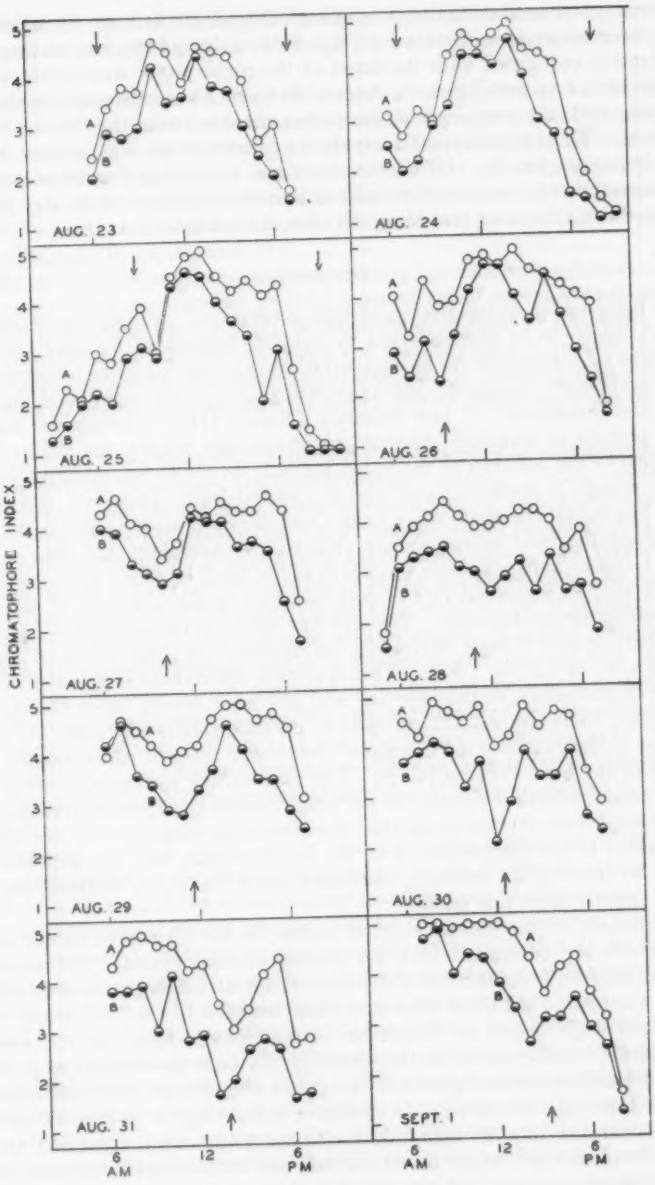
On August 20 a minimum value occurred at the time of high tide. The degree of dispersion reached, however, an over-all minimum at 8 P.M. in the diurnal rhythm of change. On August 21, when high tide occurred at 4:50 P.M., the tidal chromatophore change was obscured by the diurnally rhythmic change. The observations of August 22 were begun just following the time of high tide at 5:40 A.M. A rapid increase in degree of dispersion was observed, and was found to be maintained until shortly before the time of the evening tide.

On August 23, 24, and 25 (Fig. 5) observations spanned the time of both morning and evening high tides. In general, the increase in concentration typical of the time of high tide appeared to be obscured by the simultaneous occurrence of the transition between the night and day phases. The expression of the tidal rhythm was apparent chiefly as the delayed occurrence of strong dispersion during the morning, and the early appearance of concentration during the afternoon. On August 24, however, a slight but nonetheless definite increase in concentration was correlated with the time of the high tide which occurred at 7 A.M.

Figure 5 also includes the observations from August 26 to September 1, inclusive. An increase in concentration was associated with the time of high tide on August 26 and again on August 27. On August 28 this characteristic tidal response, although somewhat obscured by the low degree of dispersion observed throughout the day, was again apparent. On August 29 progressively greater degrees of concentration were observed following the 7 A.M. determination, reaching



FIGURES 4–5. The melanophore behavior in the autotomized legs of crabs studied in Series III. Open circles (Curve A) represent the values observed at the time of autotomy and half-closed circles (Curve B) represent the values observed 30 minutes following autotomy. The times of high tides are indicated by arrows.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B
6 AM
12
6 PM

FIGURE 5.

a minimum correlated with the time of high tide at 11:30 A.M. A second peak occurred three hours after the time of high tide; subsequently, increasing degrees of concentration correlated with the onset of the night phase were observed.

Between 6 A.M. and 5 P.M. on August 30 there was a sustained tendency toward dispersion, with the exception of the values obtained near the time of high tide at 12:30 P.M. The characteristic increase in concentration was seen at the time of high tide on August 31. On September 1 an increasing degree of concentration was observed throughout the observations, but the minimum of the day phase values was seen during the hour preceding the time of high tide at 3 P.M.

Discussion

The behavior of melanophores of legs autotomized by light-maintained animals has been shown to be characterized by varying degrees of pigment concentration. The extent of concentration, as measured 30 minutes following autotomy, reflects the diurnal change in pigment dispersion which occurs in a population maintained in continuous illumination of low intensity (Series III). During the night phase, and the periods of transition between night and day phases, the relatively concentrated state of the melanin of the intact animals serves as one limiting value on the range of change in melanin dispersion in isolated legs. However, the relationship between the initial chromatophore values and the 30-minute values is not a parallel one. When the tidal influences are removed by averaging the data of one tidal cycle, it becomes apparent from the width of the band bounded by Curves A and B, Figure 3, that an increased rate of concentration is characteristic of afternoon and early evening determinations. The average rates of concentrations, expressed as chromatophore-unit change during the 30 minutes following autotomy, range from 0.6 for 8 A.M. to 1.2 for 5 P.M. Thus two-fold differences may exist between the behavior characteristic of the morning and afternoon hours, other factors equal.

Although there are insufficient data to exclude tidally rhythmic influences, examination of the available data suggests that lower rates of concentration are characteristic of the later evening hours. The relationship prevailing during the early morning hours, when the melanin of the intact animal is dispersing, remains obscure. It can be seen from the foregoing that the 30-minute values reflect not only the diurnal rhythm in the melanophores of the intact animal, but also a diurnal rhythm of ability to concentrate following autotomy, with the forms of these two rhythms differing slightly from one another.

Superimposed on the diurnal rhythm evident in the 30-minute values are modifications which are correlated with the phases of tidal cycles. The expression of these modifications is a function of the time of day at which the various tidal phases appear. Correlated with low tides occurring between 6 A.M. and 4 P.M. there is a characteristic high degree of dispersion in the isolated legs. In the evening this low-tide phase is antagonized by the diurnally rhythmic mechanism to produce both lessened dispersion of the pigment of the intact animals, and increased rate of concentration following autotomy. In contrast, a low degree of dispersion following autotomy near the time of high tide is characteristic whether or not the diurnal tendency for dispersion in the intact animals and maintained dispersion of melanin in isolated legs is present. During the day phase the degree of dispersion observed 30 minutes following autotomy reaches a minimum near the time of high tide,

thereby providing a method whereby the phase of the tidal rhythm of the crabs may be determined, as described in a preliminary report of these experiments (Hines, 1953).

Although in many cases a lower initial degree of dispersion can be observed around the time of high tide, the increased amount of concentration following autotomy that is associated with the time of high tide is not simply a reflection of the degree of dispersion at the time of isolation. There is a general tendency toward an increased rate of concentration near the time of high tide. This is readily apparent from Series II, and is also evident in the results of Series III (see especially the data for August 10, 12, 20, and 30).

The isolated-leg preparation provides a method for following changes in chromatophore stage resulting from the gradual inactivation of the hormonal substance or substances responsible for the stage of the chromatophores in the intact animal. It may be considered as a system in which the chromatophores remain in contact with a medium containing hormonal substances, but a medium into which the normal centers of hormone production no longer secrete additional chromatophorotropins.

Sandeen (1950) has demonstrated that a melanin-dispersing substance is present in extracts of both the sinus gland and central nervous organs. If only one such substance were responsible for the state of the chromatophores, one would expect that the various degrees of dispersion in the intact animal would be achieved by means of varying concentrations of this one substance in the blood. In this case, one would also expect that the rate of inactivation of the substance would be a function of its concentration and that, following autotomy, a specific rate of pigment concentration would be associated with each particular degree of initial dispersion. If such a simple relationship between initial degree of dispersion and rate of concentration following autotomy is not observed, it would appear that something other than a single dispersing hormone is operative in determining the state of the melanophores. The observations of Series II are illustrative in this regard. In every case, the melanin was maximally dispersed at the time of autotomy. However, the extent of concentration which occurred during the 30 minutes following autotomy varied widely.

The foregoing evidence for a dual or multiple humoral control of the melanophores is substantiated by the overlapping ranges of the 30-minute values of Series II and III. In Series III the initial intermediate degrees of dispersion were often associated with 30-minute values greater than the lowest 30-minute values observed in Series II, indicating that the various rates observed in Series II do not reflect simply inactivation of various amounts of dispersing hormone in excess of that which can be reflected in the average stage of the melanophores.

It would appear, then, quite difficult to interpret these data in terms of the action of a single dispersing hormone. The presence of a melanin-concentrating principle was first postulated by Brown and Webb (1948), and was apparently required for an adequate interpretation of the responses of *Uca* to changes in illumination (Brown and Hines, 1952) and to photoperiod (Brown and Stephens, 1951). These experiments were interpreted in terms of the role of both a dispersing and a concentrating hormone in determining the normal day-to-night and night-to-day changes. Subsequently, the degree of pigment dispersion during the day phase was found to be

correlated with the phases of the tidal rhythm (Brown, Fingerman, Sandeen and Webb, 1953). The present study of the behavior of melanophores in autotomized legs suggests that the ratio of effective concentrations of dispersing and concentrating hormones must be influenced by the tidally rhythmic mechanism, as well as by the diurnally rhythmic one. If two antagonistic hormones are responsible for determining the initial degree of dispersion, it becomes possible for any given intermediate degree of dispersion to be the result of a wide range of concentrations of one of the substances in the presence of directly varying amounts of a second. The diurnal and tidal variations in rate of concentration following autotomy may then be considered as a reflection of rhythmic variations in the amounts of the two substances. It would also appear that there is a more rapid loss of dispersing than of concentrating activity.

Summary

1. The melanophores of the legs of *Uca pugnax*, following autotomy induced during the day phase of the diurnal rhythm, tend to concentrate their pigment. An adequate measure of the extent of concentration characteristic under any given conditions can be determined by observing the degree of dispersion 30 minutes following autotomy.

2. The degree of dispersion measured 30 minutes following autotomy reflects both a diurnal and a tidal rhythm in the behavior of melanophores in autotomized legs. During the day phase, when the melanin of the intact animals is dispersed, relatively little concentration occurs following autotomy near the time of low tide. An increased degree of concentration is observed when legs are isolated near the time of a high tide occurring during the day phase. This tidally rhythmic behavior has been observed for both a population with maximally dispersed melanin, and for one with normally varying degrees of melanin dispersion.

3. The variations in rate of concentration which have been observed cannot be explained as due solely to the inactivation of the melanin-dispersing hormone. It is necessary to assume that both the state of the melanophores of the intact animal and the character of the response following autotomy are determined by the relative concentrations in the blood of a melanin-dispersing hormone and a melanin-concentrating hormone.

LITERATURE CITED

Brown, F. A., Jr., M. F. Bennett and H. M. Webb, 1953. Endogenously regulated diurnal and tidal rhythms in metabolic rate in *Uca pugnax*. *Biol. Bull.*, **105**: 371.

Brown, F. A., Jr., M. Fingerman, M. I. Sandeen and H. M. Webb, 1953. Persistent diurnal and tidal rhythms of color change in the fiddler crab, *Uca pugnax*. *J. Exp. Zool.*, **123**: 29–60.

Brown, F. A., Jr., and M. N. Hines, 1952. Modifications in the diurnal pigmentary rhythm of *Uca* effected by continuous illumination. *Physiol. Zool.*, **25**: 56–70.

Brown, F. A., Jr., and G. C. Stephens, 1951. Studies of the daily rhythmicity of the fiddler crab, *Uca*. Modifications by photoperiod. *Biol. Bull.*, **101**: 71–83.

Brown, F. A., Jr., and H. M. Webb, 1948. Temperature relations of an endogenous daily rhythmicity in the fiddler crab, *Uca*. *Physiol. Zool.*, **21**: 371–381.

Hines, M. N., 1953. Use of autotomized legs in determining the phases of the tidal rhythm in *Uca pugnax*. *Biol. Bull.*, **105**: 375–376.

Sandeen, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator*, with special reference to their origins and actions. *Physiol. Zool.*, **23**: 337–352.

THE INFLUENCE OF SEASONAL ENVIRONMENTAL CHANGES UPON THE METABOLISM, LETHAL TEMPERATURE AND RATE OF HEART BEAT OF GAMMARUS LIMNAEUS (SMITH) TAKEN FROM AN ALASKAN LAKE

JOHN KROG

Arctic Health Research Center, Public Health Service, Department of Health, Education, and Welfare, Anchorage, Alaska

Fresh water biological studies in Alaska have until quite recently been conducted mainly by scientists visiting the Territory during the few summer months. Consequently very little has been known about the conditions under which the fresh water fauna exist throughout the seasons. Prolonged winter cold, brief summer warmth and the restrictions of the habitat caused by the dehydrating effect of extensive winter freezing are environmental factors not encountered by aquatic life in temperate regions. These conditions, however, bring out a number of problems of considerable basic scientific interest.

It has therefore been the plan of this study to examine the metabolic rate of the amphipod crustacean *Gammarus limnaeus* (Smith) for evidence of adaptive adjustments to the extreme seasonal changes typical of Alaska for comparison with the adaptations to temperature shown by aquatic animals from other regions. *Gammarus limnaeus* (Smith) was selected as a suitable subject for this study because of its wide distribution in Alaska, its importance as a fish food, and its consequent significance as a link in the chain of reproduction in fresh water. Its close taxonomic relation to other amphipods abundant in much of the northern world facilitates comparison.

Gammarus limnaeus (Smith) belongs to the genus *Gammarus* (Fabricius) and the amphipod family Gammaridae. The genus is very widely distributed in both salt and fresh water. Over 30 species are known, and of those 6 are found in North America, *Gammarus limnaeus* together with *Gammarus fasciatus* being the most common and widely distributed of the northern species; both occur in Alaskan lakes and ponds. *Gammarus limnaeus* is found throughout the United States from Maine to New Mexico (Weckel, 1907). Related species are common in fresh water throughout Siberia and Northern Europe.

Previous Investigations

The underlying proposition for this study was first expressed by Krogh (1916, p. 101): "One would expect that animals living at a very low temperature should show a relatively high standard metabolism at that temperature compared with others living normally at a high temperature." The abundance and activity of life in cold northern waters in which poikilotherms from warmer waters would be in-

jured or slowed to ineffectiveness by cold is in good agreement with this basic statement.

Considerable information is available on the reaction of poikilotherms to changes in temperature and oxygen tension during experiments in the laboratory. Less attention, however, has been given to the matter of determining 1) whether certain physiological adjustments of transitory or more permanent nature take place in cold blooded animals when in their natural surroundings, and 2) to what extent these adjustments might help the animal overcome changes in the environment that otherwise could have an adverse effect upon its success in living in the habitat.

Although only a few investigations seem to have been specifically directed toward the detection of seasonal or local variation in the metabolism of aquatic poikilotherms, it is not uncommon to find references to such differences in the temperature-metabolism relation of cold blooded animals. Cronheim (1911) considered that analysis of Knauthe's (1898) experiments with carp showed seasonal variation in metabolic rate. Wells (1935) found that the Pacific killifish (*Fundulus parvipinnis*) showed variations in its metabolic rate during the year and connected the fluctuations with the yearly changes in temperature of the waters in which they lived. Bruce (1926) carried out a year's study on *Mytilus edulis*, and found a seasonal variation in the oxygen requirement of this species which he associated with the reproductive cycle. Haugaard and Irving (1943) found such small seasonal variations in the oxygen consumption of the cunner (*Tautogolabrus adspersus* Walbaum) that the slightly higher oxygen consumption in winter than in summer at each temperature below 15° C. appeared too small to be regarded significant as an adaptation. In the sand crab (*Emerita talpoidea* Say), Edwards and Irving (1943) found a change in the relation of oxygen consumption up to four times higher in winter at 30° C. than in summer. The higher oxygen consumption rates in winter they interpreted as an adjustment by means of which the animals sustained activity and growth at the low winter temperatures.

Variation in the metabolism and such related functions as heart beat and pleopod movements in animals apparently identical systematically but taken from different latitudes has been reported by several authors, *viz.* Fox (1936, 1938, 1939), Fox and Wingfield (1937), Wingfield (1939), Spärck (1936) and Thorson (1936). The general assumption has been that the different temperatures of the two environments are responsible for the differences observed between the two groups in physiological response to changes in temperature during the experiment.

Marshall, Nicholls and Orr (1935) found seasonal differences in heat tolerances of *Calanus finmarchicus* with elevation of the lethal temperatures from 24° C. winter to 26° C. summer.

Hörstadius (1915) noted the point of normal development for *Paracentrotus lividus* eggs and found it to vary with the season. Runnström (1936) noted similar adjustment of both the upper and the lower limits for the development of the tunicates *Ciona intestinalis, Phallusia mammillata* and *Ascidia mentula*, and the sea urchins, *Paracentrotus lividus* and *Arbacia aequituberculata*. Fry (1947) in his paper on the effects of the environment on animal activity gives an extensive review of the existing literature in this field, in which he points out the importance of considering the environmental factors in physiological research and discusses the problems of acclimatizations.

Scholander, Flagg, Walters and Irving (1953) reviewed the literature of adaptation to cold and from their own observations compared the metabolism of arctic and tropical animals, including in their list terrestrial as well as aquatic poikilothermic forms. A general summary of their findings was that arctic aquatic forms in their accustomed temperature near 0° C. maintained a metabolic rate ⅓ to 1/10 as great as the tropical forms in their usual temperature of 30° C. If, however, the metabolic curve of the tropical forms is extrapolated down to 0° C. it represented only 1/30 to 1/40 of their metabolism in their tropical environment. Arctic forms showed a considerable, although not complete, adjustment of metabolism to their environment. In terrestrial forms, on the other hand, practically no such adjustment appeared (Scholander *et al.*, 1953).

The previous findings and reports thus all indicate the ability of aquatic poikilotherms to adjust themselves to their surroundings and also show the necessity for taking into consideration the original environment of the test animals in physiological experiments. From these former findings it could be expected that the amphipods from an Alaskan lake would show a higher oxygen consumption at a given low temperature in winter than in summer, with a higher metabolism that could facilitate a higher rate of growth in the winter months than would be expected from their temperature-metabolism relation in summer. In order to regard physiological processes as adaptive it is of prime importance to refer them to the conditions of the animal's natural environment and their behavior during the cycle of the seasons.

Material

The animals used in the experiments were all taken from Goose Lake, an eutrophic-type lake about two miles south of Anchorage, Alaska, averaging about eight feet in depth and covering about 30–40 decares. The margins of the lake are swampy, and there is no definite inlet or outlet. The banks contain deposits of peat over gravel and the bottom of the lake consists of a 1½-foot deep layer of mud and decomposing material, a so-called false bottom underlain by a base of glacial gravel. In summer the lake has a rich vegetation of water lilies, *Nymphaea,* and other water plants, with water temperatures rising to more than 20° C. In winter the lake freezes to a depth of about 3–3½ feet or more. It is doubtful, however, that it ever freezes to the bottom. Ice is on the lake from the middle part of October to the first part of May.

The animal life of the lake is surprisingly rich, with many insect larvae and a number of crustaceans including many cladocera and smaller numbers of amphipods and copepods. Snails, leeches, flatworms, and mites are also common. In winter the amphipods seem more abundant and a smaller number of cladocera and copepods are present. Whirligig beetles (Gyrinidae), boatmen (Corixidae), and water beetles (Dysticidae) are found as adults, and snails, flatworms, leeches, and the larvae of dragonflies (Aeshnidae), and damselflies (Agrionidae), are commonly found on the bottom.

Only one species of amphipod was found, *Gammarus limnaeus* (Smith), described by S. I. Smith (1874). The animals were present in the lake in fairly large numbers throughout the year. The main breeding season of *G. limnaeus* seems to be shortly after breakup of the ice in the spring. At that time they are abundant

along the shores, where many are found copulating in the shallow water. Some ice may still remain in the middle of the lake. The number of *G. limnaeus* seems to fluctuate through the summer, varying according to location.

The *G. limnaeus* used for study were caught with a dip net from the shore or from a rubber raft in summer, and in the winter through a three-foot square hole in the ice which was allowed to freeze over to keep the water from contact with air. In the laboratory the amphipods were kept in large thermos jars at as near the lake temperature as possible. Animals were usually caught in the morning and observations were started immediately, thereby avoiding prolonged exposure to indoor conditions and significant changes in temperature. The animals used were of various sizes and of both sexes as no significant sex differences in oxygen consumption were observed. Animals infested with parasites, such as acanthocephalid larvae and what was probably a larval tapeworm, were eliminated. All animals carrying eggs or showing low vitality were discarded.

Experimental Procedure

1. O_2 consumption. Oxygen consumption was measured by means of Scholander's micro- and semi-micro respirometers (Scholander, 1950). The temperatures in the water baths were kept constant within plus or minus 1° C. During most of the experiments simultaneous runs at four different temperatures, ranging from 0° C. to 22° C., were performed so as to utilize animals with as similar physiological background as possible. Fifteen individuals in separate chambers were used at each temperature and necessary handling was done by means of a pipette so as to avoid damaging the fragile legs or respiratory organs. Filtered lake water was used in the respiratory flasks. Blanks with lake water were run parallel to the experiments in the same water bath. Measurements were timed from several hours up to 24 to secure representative data for the oxygen consumption. Day and night measurements and those conducted inside a dark, walk-in ice box revealed no significant diurnal cycles or influence of light. Data obtained on animals that died during one observation and on those showing low vitality were discarded.

2. Adequacy of oxygen supply. Consideration was given to the possible influence of changing oxygen tension upon the metabolic rate of invertebrates, as reported by Amberson, Mayerson and Scott (1924). Analysis of the water from the vials after the termination of the measurements revealed that the animals themselves, by their respiratory movements and activity, stirred the water sufficiently to keep the oxygen content of the shallow water in the vials at more than ⅔ saturation. Analysis of ½ $cm.^3$ of the water from the respiration vessels was made by means of the Winkler method modified according to van Dam (1935), and conducted inside a syringe. Titration was carried out with Scholander's microburette (Scholander, Edwards and Irving, 1943), using a magnetic stirring device operating as described by Linderström-Lang and Holter (1940).

3. Weight and composition of specimens. After the runs the animals were blotted with a filter paper and dried to constant weight without heat in a desiccator with calcium chloride, to determine the water content and the dry weight of the animals. A determination of the hydrolyzable material of the animals was carried out to facilitate comparison with similar figures cited in the literature. For this purpose a group of fifty animals was dried with filter paper, weighed, and dried in a

desiccator in the same way as for determination of dry weight. After weighing, the animals were boiled in 10% KOH for one hour and the residual matter filtered, dried and weighed. Residue, principally chitinous and similar materials undissolved in KOH, might be considered inert in ordinary respiration. The weight of

TABLE I

Content of water and hydrolyzable material of Gammarus limnaeus (Smith)

Date	Wet wt. of 50 animals grams	Dry wt. of 50 animals grams	Water %	Hydrolyzable material of 50 animals grams	Hydrolyzable material of 50 animals %
1–9–51	1.020	0.240	79.6		
1–15	1.010	0.215	78.8		
1–23	1.020	0.225	77.9		
3–12	0.860	0.165	80.8	0.816	14.0
3–26	0.782	0.143	81.8		
3–28	0.850	0.160	81.2		
4–2	0.922	0.167	81.8		
4–4	0.927	0.165	82.2		
4–9	1.076	0.184	82.9	1.016	11.5
4–12	0.994	0.174	82.5	0.938	11.8
4–16	0.975	0.165	83.1		
5–7	0.948	9.194	79.5		
5–10	0.972	0.194	80.1		
5–16	1.067	0.199	81.3		
5–24	1.057	0.214	79.8		
6–7	1.126	0.233	79.3		
6–11	1.035	0.195	80.1		
6–21	1.465	0.265	81.9		
7–10	1.025	0.207	80.3		
9–20	1.110	0.220	80.2		
9–26	1.060	0.215	79.7		
10–8	1.237	0.240	80.8		
10–22	1.635	0.320	78.6		
11–21	1.412	0.315	79.1		
12–6	1.390	0.257	81.5		
12–13	1.750	0.352	79.8		
12–20	1.557	0.332	78.5		
1–9–52	2.020	0.410	79.7		
1–17	1.377	0.281	79.5		
2–5	1.698	0.349	79.4		
4–8	1.812	0.287	84.3		
Average			80.5	0.923	12.4

the hydrolyzable material was calculated in per cent of wet and dry weight (see Table I). There was no suggestion that this residue changed sufficiently to alter results. The reference of oxygen consumption to wet weight, dry weight or hydrolyzable material should therefore give about the same relationship. The oxygen consumption was most conveniently given per unit wet weight and was chosen as a basis for the figures used in the accompanying tables and graphs.

4. "Lethal" temperature. Determinations of the "upper lethal" temperature at the end of the winter and in the summer were made. The highest tolerable temperatures were determined by suspending small glass vials containing lake water and 5 animals each in thermos bottles filled with water with a temperature difference of 2° C. from 24° C. to 36° C. The vials were checked every 15 minutes and the dead animals were picked out. Paralyzed animals showing no signs of life were counted together with the dead ones even though a few recovered when placed in cold water. Any attempt to distinguish between paralyzed and dead animals would be very difficult and possibly irrelevant. One hundred per cent survival after one hour exposure to a given temperature was used as a standard for determining the animal's level of heat tolerance.

5. Heart rate. The heart beat was counted by means of transmitted light that had passed through a ¾-inch thick layer of flowing water in a specially constructed water-cooled microscope stand (Krog and Simmet, 1954) which permitted temperature control to within plus or minus 0.1° C. The animals were surrounded by lake water during the test. The temperature of the water immediately surrounding the animals was measured by means of a fine thermocouple. Four to five ½-minute counts were taken at each temperature and the average rate of heart beat calculated.

Results of Observations and Experiments

Temperature and oxygen supply in the lake. The curves in Figure 1 show the yearly variation in temperature and oxygen content of the water in Goose Lake, together with the saturation curve for oxygen dissolved in the water at the recorded temperatures. The oxygen content in the lake reached its lowest values in February and March when it dropped below amounts detectable with the Winkler method used for this determination (Standard Methods for Examination of Water and Sewage, page 724). The summer months give quite steady values for dissolved oxygen. As seen from the curve, the increase of oxygen in the lake starts before the breakup of the ice. This is probably due to accumulation of water from melting snow on the top of the ice, which is later drained into the lake through enlarged cracks. This incident is followed by a lifting of the ice caused by the rising water level and subsequently by appearance of open water along the shores. From this time on, direct contact between the lake water and the air is established, and the O_2 content increases gradually.

The water in the lake, however, never reached saturation at any temperature. After the oxygen content reached a value of around 8–9 ppm., it leveled off and remained at that level throughout the summer. The first decrease of oxygen in the lake followed, after some delay, the freeze-up in the fall and it continued to decrease at a fairly even rate in the following months until it reached its minimum in February, March, and April. Reduction of the oxygen supply in winter is probably

common in the shallow lakes of Alaska and might explain why no fish have been found in so many of the smaller, shallow, non-circulated lakes although fish food appears to be abundant.

The temperature of the water in the lake also varied considerably with the season, rising in summer above 20° C. (see Fig. 1). The temperature increased rather sharply in the spring because of the shallowness of the water and the lack of any significant out- and in-flow. The pH was not observed to change consistently with the season and stayed between 7.0 and 8.0.

Variation of the amphipods' oxygen consumption with size was found to occur in the course of the year as is shown when the oxygen consumption at 10° C. is

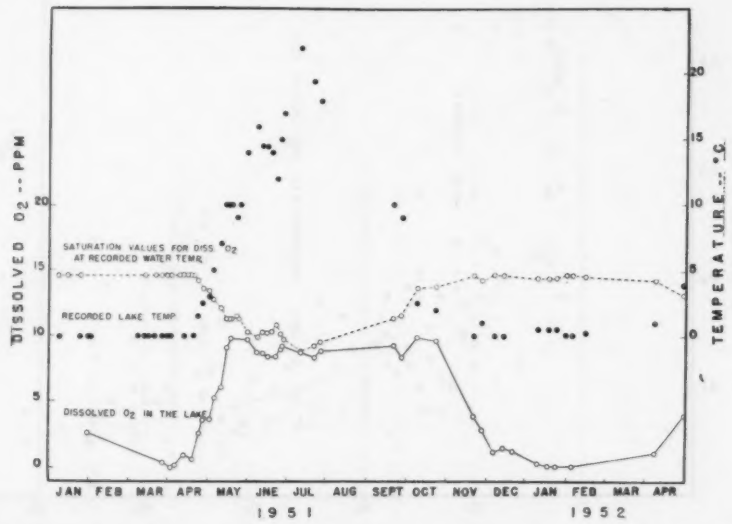
DISSOLVED O2 -- PPM
TEMPERATURE -- °C
SATURATION VALUES FOR DISS. O2 AT RECORDED WATER TEMP.
RECORDED LAKE TEMP.
DISSOLVED O2 IN THE LAKE
JAN FEB MAR APR MAY JNE JUL AUG SEPT OCT NOV DEC JAN FEB MAR APR
1951
1952

FIGURE 1. Recorded temperature in Goose Lake 1951 through April 1953, together with calculated saturation values for dissolved oxygen at those temperatures, and actual recorded dissolved oxygen during the same period, measured in parts per million by weight.

plotted against weight (see Fig. 2). Curves plotted at other temperatures show similar although not so pronounced variation. A further breakdown of the data in weight groups was not found desirable as it would complicate the final comparison between summer and winter animals and only slightly improve the spread of the values.

To simplify comparison, the year was divided into two seasons—June and July —(see Fig. 3) representing the typical summer period with fairly high temperatures and rather constant oxygen content of the water, and the late winter—February through March—which is the period of lowest oxygen content, disregarding the transition between these two periods. During the winter period a significant lowering of the oxygen utilization of the animals was found to occur when the oxygen content in the lake water was lowest, suggesting a difference between the

seasonal adjustment in these animals and the Crustacea previously investigated. These have, however, been mainly marine forms or those from well circulated waters with fairly high and stable oxygen tensions and with the temperature as the most important variable.

Figure 4 shows the upper temperature tolerance as determined for the animals in winter as well as in summer. The highest temperature which the animal can tolerate as determined by 100% survival for one hour, seems to be about 26° C. for the winter animals, and for the summer animals from 30°–32° C. This shows a lowering of the resistance to heat by 4°–6° C. in the cold season as compared to the summer, which is about what would be expected from previous investigations of temperature adjustments in invertebrates.

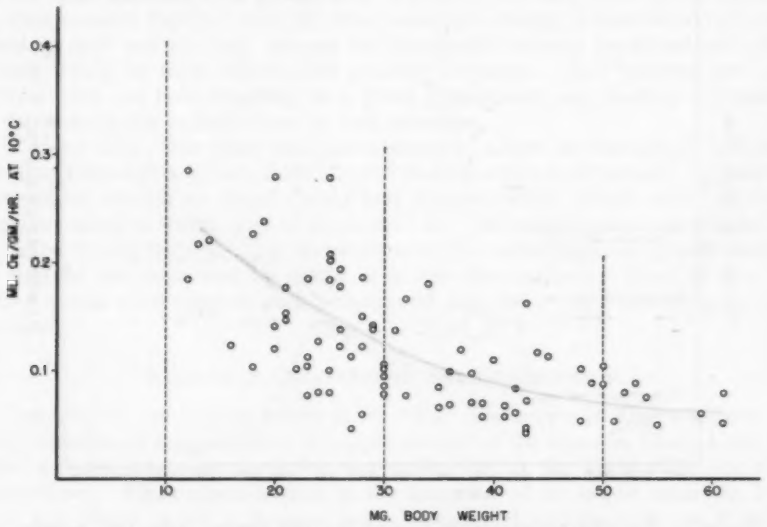

FIGURE 2. Relation between body weight and oxygen consumption at + 10° C.

The pulsation rate of the "heart," or more correctly the dorsal vessel of the animal, was recorded at the end of winter (March) and at the end of summer (the last part of August) in order to determine the extent of seasonal variation. Figure 5 shows the rate of pulsation plotted against temperature. A slight elevation in the relation of heart rate to temperature seemed to occur in winter with generally lower heart rates at all temperatures for the summer animals. Above 19° C. the winter animals were observed to show irregularity in their "heart" beat with an eventual decrease in rate. The larger spread in the data for the winter animals at the higher temperature as compared with the summer animals is believed to be mainly caused by this reaction (see Fig. 5). The summer animals on the other hand showed a normal increase of the "heart" beat above 14° C. up to around 23° C. after which irregularity and consequent gradual drop in heart rate occurred as in the winter animals.

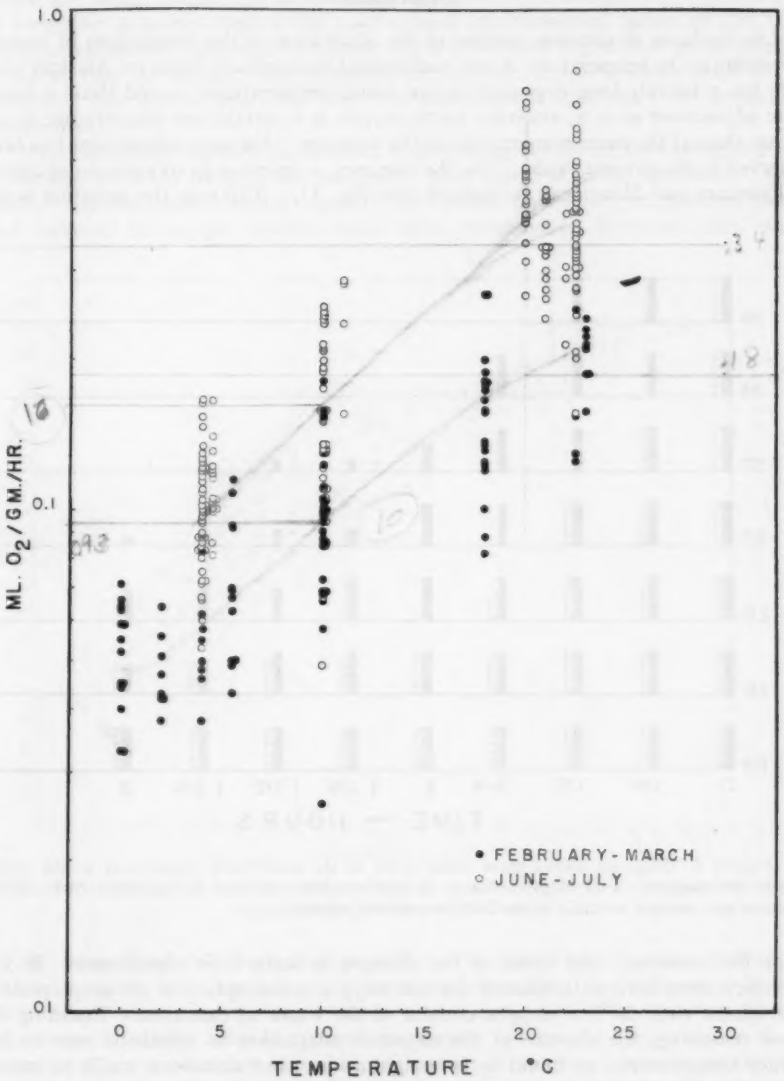

FIGURE 3. The relation between oxygen consumption and temperature in the periods February through March and June through July, plotted on a semilog scale. The animals from the two different seasons are marked with separate symbols.

Discussion

On the basis of previous studies of the adaptation of the metabolism of aquatic invertebrates to temperature, it was anticipated that animals from an Alaskan lake, with the relatively long exposure to low water temperatures, would show a metabolic adjustment so as to consume more oxygen at a certain low temperature in the winter than at the same temperature in the summer. No such adjustment has been observed in the present study. On the contrary, a decrease in oxygen consumption in February and March can be noticed (see Fig. 3). Although the variation is not

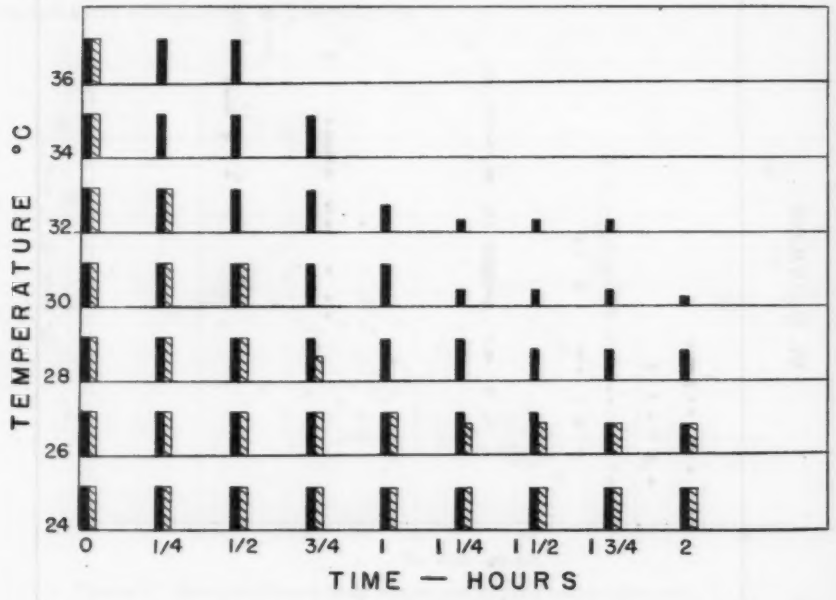

Figure 4. Diagram shows upper lethal level of the amphipods respectively in the winter and in the summer. Full length columns at extreme left represent five animals each. Filled columns are summer animals, cross lined are winter animals.

large the constancy and trend of the changes indicate their significance. It will therefore seem natural to connect the low oxygen consumption of the amphipods in late winter with the low oxygen content of the water at that time. Pursuing the same reasoning, the absence of the expected adaptation in metabolic rate to low winter temperatures, as found by investigators in other situations, could be caused by the fact that there is not enough oxygen for such an adaptation to be useful.

Assuming that adjustments made by animals in the face of unfavorable environmental factors are directed toward the factors that are most critical, the differences between previous findings and the situation reported at Goose Lake can be more easily understood. The low oxygen consumption of the animals from Goose Lake

in the late winter might thus be described as a result of a compensatory adjustment in metabolic economy toward the most critical environmental factor at that time. Such an adjustment, although not large, might be of vital importance to the animals, living as they do through long periods with oxygen supplies which must be approaching the lowest limit that they can tolerate and still carry on an aerobic metabolism.

Experimentally induced adjustment to low oxygen content by exposure to environment with low oxygen has previously been reported in the literature. Lund (1921) reported that repeated exposure of *Planaria* to low oxygen tensions caused less increase in oxygen uptake after each experiment. Hiestand and Singer

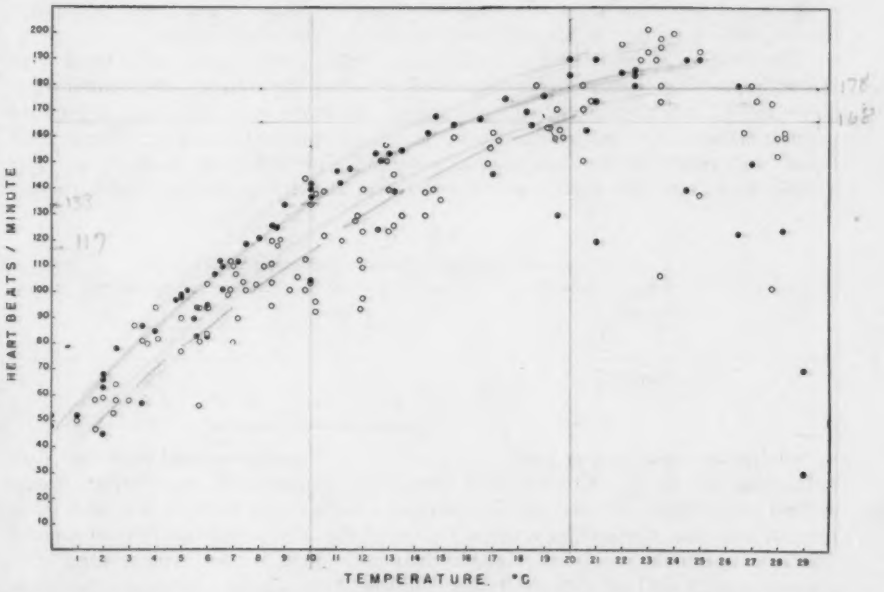

FIGURE 5. Heart rate in beat per minute plotted against time. Open circles represent summer animals and solid dots, winter animals.

(1934) also found that leeches responded by "acclimatization" after repeated exposure to water with low oxygen content, and that they gradually went back to their former state after the experiments were terminated. The question whether these experimentally induced changes in oxygen uptake are comparable with the natural reduction occurring in amphipods cannot be answered but the similarity is striking. On the other hand the benefit of such an adjustment for the amphipods seems quite clear and the remarkable ability of the animals to get along with the low amount of oxygen available in the late winter can be more easily understood. Adjustment to a high oxygen consumption in the winter, such as that reported in literature dealing with other poikilotherms, would in the case of the Alaskan amphipods

be unfavorable as it is of more moment for them to survive the winter than to maintain a high metabolism when the oxygen supply is critically scant.

The Q_{10} as calculated from Figure 3 shows values around 2, the Q_{10} in the winter being slightly lower in value than in the summer (see Table II). Scholander *et al.* (1953) have given a complete discussion of the problem of Q_{10} as an indication of an animal's state of adaptation to the environment. They concluded that physiological adaptation of poikilotherms to cold has its manifestation in the lateral displacement of the metabolism temperature curve but not in the change of its slope as expressed as change in Q_{10}. In the amphipods the summer and winter values for Q_{10} did not differ appreciably.

The changes in the upper lethal temperature levels follow fairly closely those expected on the basis of previous reports upon temperature tolerances of poikilotherms, adding no new information regarding this type of adjustment.

The changes in heart beat, although quite small, show on the other hand some rather interesting trends. The differences between the summer and winter rates above 19° C. indicate that the summer animal can increase its heart rate beyond the point at which the winter animal's heart ceases to function normally. The over-all lower heart rate of the summer animal below 20° C. is not easily understood. Fox (1939) found that the marine polychaete worm *Perinereis cultiifera* from English

TABLE II

Q_{10} of O_2 consumption in Gammarus limnaeus (Smith)

Time	Temperature °C.		
	0–10	5–15	10–20
Summer	—	2.4	2.0
Winter	2.1	2.1	1.8

waters had the same rate of heart beat at 14° C. as the same animal from the Mediterranean had at 20° C. He also found that crustaceans from colder waters around the British Isles had, on the average, a higher rate of heart beat at a given temperature than those from warmer waters of the Mediterranean despite the fact that arctic animals are usually bigger than individuals of the same species from warmer waters, and an opposite relationship between size and heart rate seems to exist commonly among invertebrates. The present observation of changes in heart rate according to season within the same animal population could be compared to the findings reported by Fox (1936) on marine invertebrates taken from waters at different latitude and temperature, except that the changes observed in the Alaskan amphipods might be explained as being brought about through adjustments to the yearly variation in the environment. The particular component or components of the environment which induce this reaction, however, cannot be fully determined. A direct parallel to the cases reported upon by Fox, with an apparent relationship between the physiological responses to laboratory-imposed temperature changes and the temperature of the animal's original environment, would only be possible if the oxygen content of the water were plentiful. Under the reported conditions there is also the probability that the heart rate would increase in the winter so as to compensate for the lower oxygen content of the water at that time of year.

This and related studies suggest that considerable physiological flexibility seems to be present in individual aquatic poikilotherms, making it possible for them to make adjustment to comparatively large variations in their environments. The differences between the winter and summer temperature in Alaska equal the difference in the sea around Britain and in the Mediterranan. One population of amphipods encounters the Alaskan seasons by adaptive physiological changes occurring during the life of individuals. Two isolated sections of a population might continue to live safely within the extremes of their physiological adaptability without offering a visible hold for natural selection to differentiate their physiological processes.

It is a great pleasure for me to acknowledge the guidance, interest, and kind criticism by Dr. Laurence Irving during the course of this study. I would also like to thank Miss Mildred Wagner for the excellent help in doing determinations of dissolved O_2, and the drawings; my wife, Hildur Krog, for assistance in the calculation of the data; Dr. P. F. Scholander for kindly reviewing the manuscript and for valuable suggestions concerning the presentation of the results, and Dr. Leslie Hubricht for identifying the amphipods.

Summary

1. A year's cycle of the oxygen consumption of the amphipod, *Gammarus limnaeus* (Smith), has been recorded. The metabolism has been related to the environmental factors encountered in a shallow Alaskan lake, which is ice-covered during seven winter months.

2. A seasonal change in the metabolic rates of the animals accompanied changes in the available oxygen. These resulted in decreased oxygen consumption during the winter when the oxygen supply in the lake was low.

3. The lethal temperature of the amphipods changed from 26° C. in winter to 30°–32 C. in summer.

4. The rate of the heartbeat of the animals varied slightly with the season, with a higher average rate at temperatures below 20° C. in winter than in summer.

LITERATURE CITED

Amberson, W. R., H. S. Mayerson and W. J. Scott, 1924. The influence of oxygen tension upon metabolic rate in invertebrates. *J. Gen. Physiol.*, **7**: 171–176.

American Public Health Association, 1926. Standard methods for the examination of water and sewage. American Public Health Association.

Bruce, J. R., 1926. The respiratory exchange of the mussel, *Mytilus edulis* L. *Biochem. J.*, **20**: 829–846.

Cronheim, W., 1911. Gesamtstoffwechsel der kaltblütigen Wirbeltiere im besonderen der Fische. *Zeitschr. f. Fischerei.*, **15**: 319–370.

Edwards, G. A., and L. Irving, 1943. The influence of temperature upon the oxygen consumption of the sand crab, *Emerita talpoida* Say. *J. Cell. Comp. Physiol.*, **21**: 169–182.

Fox, H. Munro, 1936. The activity and metabolism of poikilothermal animals in different latitudes. I. *Proc. Zool. Soc. Lond., A*, 945–955.

Fox, H. Munro, 1938. The activity and metabolism of poikilothermal animals in different latitudes. III. *Proc. Zool. Soc. Lond., A*, **108**: 501–505.

Fox, H. Munro, 1939. The activity and metabolism of poikilothermal animals in different latitudes. *Proc. Zool. Soc. Lond., A*, **109**: 141–156.

Fox, H. Munro, and C. A. Wingfield, 1937. The activity and metabolism of poikilothermal animals in different latitudes. II. *Proc. Zool. Soc. Lond., A*, **107**: 275–282.

FRY, F. E. J., 1947. Effects of the environment on animal activity. Univ. Toronto Studies Biol. 55, *Pub. Ont. Fish. Res. Lab.*, **68**: 1–62.

HAUGAARD, NIELS, AND L. IRVING, 1943. The influence of temperature upon the oxygen consumption of the cunner (*Tautogolabrus adspersus* Walbaum) in summer and in winter. *J. Cell. Comp. Physiol.*, **21**: 19–26.

HIESTAND, W. A., AND J. SINGER, 1934. Certain factors influencing the respiratory metabolism of the leech (*Hirudo medicinalis*). *Proc. Ind. Acad. Sci.*, **43**: 205–210.

HÖRSTADIUS, S., 1915. Temperaturanpassung bei den Eiern von *Paracentrotus lividus* (Lk.). *Biol. Gen.*, **1**: 522–536.

KNAUTHE, K., 1898. Zur Kenntnis des Stoffwechsels der Fische. *Pflüg. Arch. ges. Physiol.*, **73**: 490–500.

KROG, J., AND R. SIMMET, 1954. An attachment for the low-power binocular microscope facilitating observations at controlled temperatures. *Trans. Amer. Micr. Soc.* In press.

KROGH, A., 1916. The respiratory exchange of animals and man. Longmans, Green and Co., London.

LINDERSTRÖM-LANG, K., AND H. HOLTER, 1940. Die enzymatische Histochemie. *In:* Bamann, E., and Myrbäck, K., Die Methoden der Fermentforschung. Thieme, Leipzig, 1132–62.

LUND, E. J., 1921. Oxygen concentration as a limiting factor in the respiratory metabolism of *Planaria agilis*. *Biol. Bull.*, **41**: 203–220.

MARSHALL, S. M., A. G. NICHOLLS AND A. P. ORR, 1935. On the biology of *Calanus finmarchicus*. Part VI. Oxygen consumption in relation to environmental conditions. *J. Mar. Biol. Ass. U.K.*, **20**: 1–27.

RUNNSTRÖM, S., 1936. Die Anpassung der Fortpflanzung und Entwicklung mariner Tiere an die Temperaturverhältnisse verschiedener Verbreitungsgebiete. *Bergens Mus. Aarb.* No. 3.

SCHOLANDER, P. F., 1950. Volumetric plastic micro-respirometer. *Rev. Sci. Instrum.*, **21**: 378–380.

SCHOLANDER, P. F., G. A. EDWARDS AND L. IRVING, 1943. Improved micrometer burette. *J. Biol. Chem.*, **148**: 495–500.

SCHOLANDER, P. F., W. FLAGG, V. WALTERS AND L. IRVING, 1953. Climatic adaptation in arctic and tropical poikilotherms. *Physiol. Zool.*, **26**: 67–92.

SMITH, S. I., 1874. The crustacea of the fresh waters of the United States. *Rep. U.S. Comm. Fish.*, **2**: 623–665.

SPÄRCK, R., 1936. On the relation between metabolism and temperature in some marine lamellibranches, and its zoogeographical significance. *K. danske vidensk. Selsk.*, **13**: 3–27.

THORSON, G., 1936. The larval development, growth and metabolism of arctic marine bottom invertebrates. *Medd. Grønland., 100, No. 6.*

VAN DAM, L., 1935. A method for determining the amount of oxygen dissolved in 1 cc. of water. *J. Exp. Biol.*, **12**: 80–85.

WECKEL, A. L., 1907. The fresh-water amphipoda of North America. *Proc. U.S. Nat. Mus.*, **32**: 38–49.

WELLS, N. A., 1935. Variations in the respiratory metabolism of the Pacific killifish, *Fundulus parvipinnis*, due to size, season, and continued constant temperature. *Physiol. Zool.*, **8**: 318–336.

WINGFIELD, C. A., 1939. The activity and metabolism of poikilothermal animals in different latitudes. IV. *Proc. Zool. Soc. Lond., A*, **109**: 103–108.

SEROLOGICAL COMPARISONS AMONG FOUR CLASSES OF MOLLUSCA [1]

CHARLES A. LEONE AND CARLON W. PRYOR [2]

Department of Zoology, University of Kansas, Lawrence Kansas

Serological comparisons of the proteins of organisms are a means of studying the results of biochemical evolution. With the precipitin test the chemical similarities of the proteins of animals can be measured quantitatively and the degrees of relationship thereby estimated. The purpose of this paper is to report on some serological comparisons of the proteins from the four Classes Amphineura, Gastropoda, Pelecypoda, and Cephalopoda in the Phylum Mollusca.

Materials and Methods

Antigens

Table I lists the species of Mollusca from which antigens were obtained. Sera from *Octopus vulgaris* and *Sepia officinalis* were collected at the Stazione Zoologica di Napoli, Naples, Italy in the summer of 1948. Serum from *Busycon caricum* was obtained at the laboratory of the United States Fish and Wildlife Service, Beaufort, North Carolina, in the summer of 1951. The other antigens were collected at the Friday Harbor Marine Laboratory, University of Washington, Friday Harbor, Washington, in the summer of 1952. 'Merthiolate' in a final concentration of 0.01 per cent (1 : 10,000) was added as a preservative at the time of collection.

Antisera

The antisera used in this study were produced in healthy, adult rabbits. Table I shows the schedule of injections followed for the production of these antisera. Multiple series of injections were given to all rabbits except the one which produced the antiserum against the proteins of *Tonicella lineata*. This rabbit received a single series of injections; seven injections were given on alternate days—1.0, 1.5, 2.0, 2.0, 5.0, 5.0, and 5.0 ml. The fifth and sixth were intraperitoneal and the others were intravenous. Each rabbit was given a trial bleeding from the median artery of the ear eight days after the last injection of a series. At the end of the injection schedule those rabbits with good levels of antibody were completely exsanguinated by intracardial puncture after a fast of 18 hours to obtain sera free from dissolved lipids. Whole blood, obtained either by bleeding from the median artery of the ear, or by intracardial puncture, was placed in lusteroid centrifuge tubes, permitted to clot, rimmed, and held at $0 \pm 2°$ C. for 1 to 10 hours. Expressed serum was

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[2] Public Health Service Predoctorate Research Fellow of the National Microbiological Institute.

TABLE I

Mollusca used in serological comparisons and injection schedules used to produce antisera

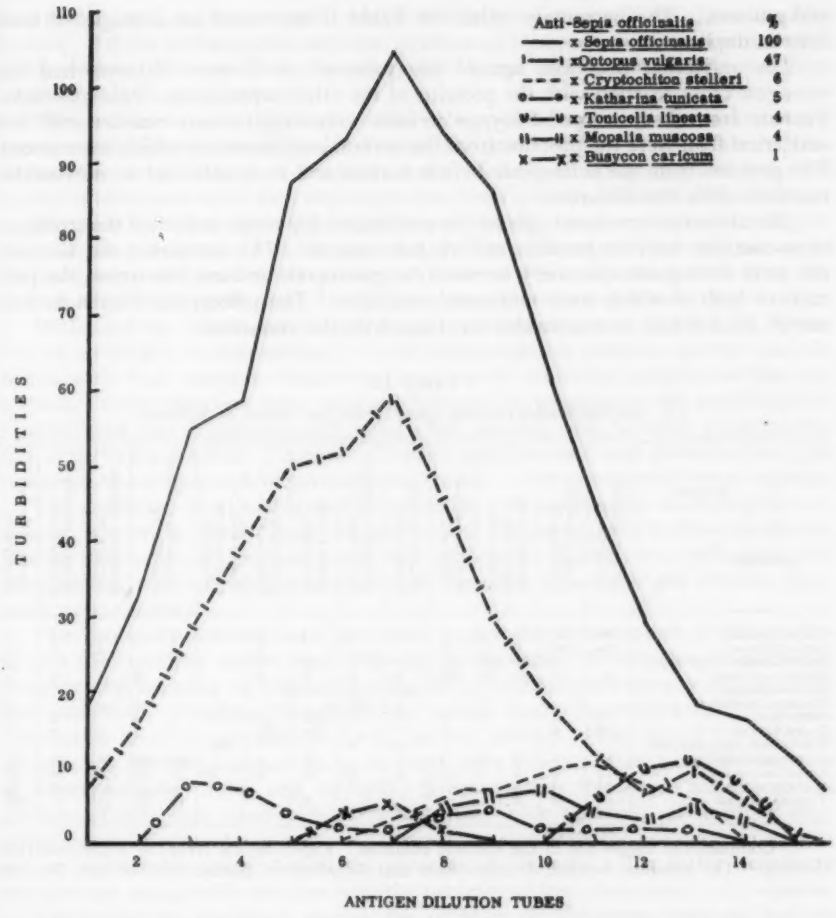
Species	Serum protein gms./100 ml.	Injection schedule	Series of injections
PHYLUM MOLLUSCA			
Class Cephalopoda			
Octopus vulgaris Linnaeus	1.75	1.0, 1.5, 2.0 ml., iv*., alt. days†	4
Sepia officinalis Linnaeus	4.20	1.0, 1.5, 2.0 ml., iv., alt. days	4
Class Gastropoda			
Argobuccinum oregonensis (Redfield)	3.10	1.0, 1.0, 2.0, 2.0 ml., iv., alt. days	2
Busycon caricum (Gmelin)	2.40	1.0 1.5 2.0 ml., iv., alt. days	4
Class Pelecypoda			
Macoma nasuta Conrad	1.27	1.0, 1.5, 2.0, 2.0 ml., iv., alt. days	2
Modiolus modiolus Linnaeus	0.27	1.0, 2.0, 2.0, 2.0 ml., iv., alt. days	2
Pecten hericus Gould	0.65	1.0, 1.0, 2.0, 4.0 ml., iv., alt. days	2
Pecten hindsii (Carpenter)	0.45	1.0, 1.0, 2.0, 4.0 ml., iv., alt. days	2
Pododesmus macroschisma Deshayes	0.47	1.0, 2.0, 2.0, 2.0 ml., iv., alt. days	2
Class Amphineura			
Cryptochiton stelleri Middendorf	2.47	2.0, 1.5, 2.0 ml., iv., alt. days	4
Katharina tunicata Wood	4.80	1.0, 1.5, 2.0 ml., iv., alt. days	4
Mopalia muscosa (Gould)	1.07	1.0, 1.5, 2.0 ml., iv., alt. days	4
Tonicella lineata Wood	0.67	1.0, 1.5, 2.0, 2.0 ml., iv., alt. days, 5.0, 5.0 ml., ip**., 5.0 ml., iv., alt. days	1

*iv = intravenously, **ip = intraperitoneally, † alt. days = alternate days

poured into clean, dry lusteroid centrifuge tubes and centrifuged for 30 minutes at 375 g. The resulting clear serum was decanted, sterilized by passage through Seitz filters, put into sterile serum vials and frozen at – 20° C. until used.

METHODS OF TESTING

Quantitative measures of precipitin reactions were made with the Libby (1938) photronreflectometer, using the methods outlined by Boyden and DeFalco (1943) and Leone (1949). Precipitin tests were performed by adding a constant amount of antiserum to doubling dilutions of antigens. The proteins were diluted with 0.9 per cent saline buffered with $M/150$ phosphate salts. 'Merthiolate' in final concentration of 0.01 per cent (1 : 10,000) was added as a bacteriostatic agent to the saline diluent. Initial dilutions of antigens were made on the basis of the known concentration of protein (Table I); dilutions ranged from 1 : 62.5 to 1 : 4,096,000. Control readings of antigen and saline, and antiserum and saline, were subtracted from the total turbidity readings. Corrected values for the antigen-antibody precipitates were plotted with the turbidities on the ordinate and the antigen dilutions on the abscissa. Representative curves are shown in Figure 1. A value proportional to the area under each curve was obtained by a summation of the turbidity values (Boyden and DeFalco, 1943). Duplicate tests yield "area" values which are within ± 3 per cent of each other. The "areas" of the homologous and heterologous reactions were used to establish the relationships among the various proteins which were compared. To compute the percentage relationship, the summation of



• FIGURE 1. Precipitin reaction curves obtained with the antiserum produced against *Sepia officinalis*. Values proportional to the areas under the curves are obtained by summating the turbidity values. Comparisons are made of the "areas" to determine the per cent relationship among the species represented.

the homologous reaction was divided into the summation of the heterologous reaction and the quotient multiplied by 100.

EXPERIMENTAL RESULTS

All antisera produced using the schedules of injections given in Table I had high levels of antibodies. Table II gives a summary of the serological relationships which were calculated from the precipitin tests performed with the various antigens

and antisera. The percentage values in Table II are based on averages of tests done in duplicate.

The antiserum produced against the proteins of *Octopus vulgaris* had the strongest cross-reaction with the proteins of the other cephalopod, *Sepia officinalis*. Proteins from the gastropod *Busycon caricum* were slightly more reactive with this antiserum than were the proteins from the several amphineurans which were tested. The proteins from the pelecypods *Pecten hericus* and *P. hindsii* had no measurable reactions with this antiserum.

The antiserum produced against the proteins of *Sepia officinalis* had the strongest cross-reaction with the proteins of *Octopus vulgaris*. This antiserum did not permit us to distinguish effectively between the gastropods and amphineurans, the proteins of both of which were measurably reactive. The pelecypods *Pecten hericus* and *P. hindsii* had no measurable reactions with the antiserum.

TABLE II*

Per cent relationships among species from four classes of Mollusca

Antigens \ Antisera	*Octopus vulgaris* Cephalopoda	*Sepia officinalis* Cephalopoda	*Argobuccinum oregonensis* Gastropoda		*Busycon caricum* Gastropoda	*Macoma nasuta* Gastropoda	*Modiolus modiolus* Pelecypoda	*Pecten hericus* Pelecypoda	*Pecten hindsii* Pelecypoda	*Pododesmus macroschisma* Pelecypoda	*Cryptochiton stelleri* Amphineura		*Katharina tunicata* Amphineura	*Mopalia muscosa* Amphineura	*Tonicella lineata* Amphineura
Octopus vulgaris	100	47	0	5	1	0	0	0	0	0	1	1	1	1	0
Sepia officinalis	69	100	0	3	0				0		2		8	0	0
Argobuccinum oregonensis		0	100	100	33			0	0		0		0		0
Busycon caricum	21	1	58	34	100	0	0	0	0		7	0	2	0	0
Macoma nasuta						100	0			0					
Modiolus modiolus				2		0	100			13		0	0		0
Pecten hericus	0	0	0	3	0	22	4	100	56	0	0	0		0	0
Pecten hindsii	0	0	0		0			74	100			0	0	0	
Pododesmus macroschisma						0	5			100					
Cryptochiton stelleri	16	6	0	0	2			19	6		100	100	79	65	31
Katharina tunicata	14	5	0	1	12	0	0	19	0	0	54	80	100	69	25
Mopalia muscosa	13	4	0								36	65	63	100	26
Tonicella lineata		4	0		0			0			10	20	24	23	100

* Comparative values are in the vertical columns. Experimental error: ± 3 per cent relationship. The columns headed *Argobuccinum* and *Cryptochiton* contain sets of data for two antisera.

The two antisera which were produced against the proteins of the gastropod *Argobuccinum oregonensis* were specific with respect to cross-reactivity with the proteins from representatives of the other Classes in the Phylum Mollusca. Both antisera had strong cross-reactions with the other gastropod, *Busycon caricum*. The less specific of the two antisera was slightly reactive with the proteins from the cephalopods *Octopus* and *Sepia*, the pelecypods *Modiolus* and *Pecten* and the amphineuran *Katharina*. The more specific of the two anti-*Argobuccinum* sera gave measurable cross-reactions only with the proteins of the other gastropod *Busycon*.

The proteins of the other gastropod, *Argobuccinum*, were most reactive with the antiserum to *Busycon caricum*. The reactivity of the proteins of the amphineuran *Katharina tunicata* with this antiserum was strong as compared to the slightly posi-

tive results, and negative results obtained with the proteins of the other amphineurans. Of the cephalopods, only the proteins of *Octopus* were reactive and to a small extent. Proteins from the pelecypod *Pecten* had no measurable reactivity with the anti-*Busycon* serum.

The antiserum to the proteins of the pelecypod *Macoma nasuta* was strongly reactive with the proteins of *Pecten hericus,* but gave negative results with the proteins of the other pelecypods *Modiolus* and *Pododesmus.* Negative also were the results of tests performed with representatives from the other Classes in the Phylum.

The anti-*Modiolus* serum was slightly, and equally, cross-reactive with the proteins of the pelecypods *Pododesmus* and *Pecten.* Proteins from the pelecypod *Macoma* were not measurably reactive. Proteins from representatives from the Gastropoda, Cephalopoda, and Amphineura were likewise not measurably reactive.

Proteins from *Pecten hericus* and from *Pecten hindsii* each induced the formation of strikingly similar antisera. Good differentiation between species was obtained with both antisera when they were tested with the proteins of the two pectens. Cross-reactions were obtained between the proteins of the amphineurans *Cryptochiton* and *Katharina* and anti-*Pecten hericus,* and between *Cryptochiton* and anti-*Pecten hindsii.* Proteins of other amphineurans and proteins from the cephalopods and gastropods gave negative results when tested with these antisera.

The antiserum produced against the proteins of the pelecypod *Pododesmus macroschisma* was cross-reactive with the proteins of the pelecypod *Modiolus modiolus.* Results obtained with proteins from tests performed between this antiserum and the proteins from species representative of the other Classes of the Phylum Mollusca were negative.

The two antisera which were produced against the hemocyanins of the amphineuran *Cryptochiton stelleri* had differing specificities. Within the Amphineura, however, the sequence of relationship for cross-reacting proteins was the same for each antiserum. Proteins from *Katharina tunicata* had the strongest cross-reaction, followed by *Mopalia muscosa* and then *Tonicella lineata.* With one of the antisera, the proteins of the gastropod *Busycon* were more reactive than were the proteins of the cephalopods *Sepia* and *Octopus*; however, with this same antiserum, the proteins of the other gastropod, *Argobuccinum,* were negative. The other antiserum against *Cryptochiton* had a slight cross-reaction with the proteins of *Octopus* and no measurable reaction with the proteins of *Busycon.* The latter antiserum was also not measurably reactive with the proteins of the pelecypods.

The antiserum produced against the proteins of *Katharina tunicata* had its strongest cross-reaction with the proteins of *Cryptochiton. Mopalia* and *Tonicella* were next most reactive in that order. Of the species from other Classes the proteins of the cephalopod *Sepia officinalis* were most reactive. The proteins of the cephalopod *Octopus* were reactive to a lesser extent, as were the proteins of the gastropod *Busycon.* The proteins of the pelecypods had no detectable reactions with this antiserum.

Proteins from *Katharina tunicata* and *Cryptochiton stelleri* were approximately equally reactive with the anti-*Mopalia* sera and more so than were the proteins of *Tonicella lineata.* The proteins of *Octopus vulgaris* were slightly cross-reactive with this antiserum.

Reactions involving the antiserum produced against the proteins of *Tonicella lineata* were restricted to the proteins of the amphineurans. By means of the anti-

serum we were unable to distinguish effectively among the proteins of the species *Cryptochiton stelleri, Katharina tunicata,* and *Mopalia muscosa.*

Discussion

Only a few reports in the literature provide serological data on interclass relationships among the mollusks.

Galli-Valerio (1916) produced antisera against tissue-extracts of the pelecypod *Anodonta anatina* (Linnaeus). His only heterologous precipitin tests were with similar extracts from the gastropod *Limnaea stagnalis* (Linnaeus), which produced precipitates.

Erhardt (1931) produced antisera against the gastropods *Arion empericorum* Ferussac and *Limnaea stagnalis* (Linnaeus). The stronger anti-*Arion* serum cross-reacted well with the proteins of the other gastropods, but gave negative results in tests with the proteins of the amphineuran *Chiton marginatus* Pennant.

Makino (1934) employed precipitin, complement fixation and anaphylactic reactions with saline-extracted antigens from the pelecypods *Arca inflata* Reeve, *Cytherea meretrix* Linnaeus, *Ostrea gigas* Thunberg, *Paphia philippinarum* Adams and Reeve, the gastropods *Haliotis gigantea* Chemnitz, *Rapana thomasi* Crosse, *Turbo coronatus* (Gmelin) and the cephalopods *Sepiella japonica* Sasaki, and *Polypus* [= *Octopus*] *variabilis* Sasaki. Within each Class the cross-reactions between antigens and antibodies were strong. Cross-reactions between reagents which were representative of different Classes of mollusks were weak or doubtful, or gave negative results. His precipitin data were extensive and he judged that they indicated the gastropods resembled the pelecypods more than they did the cephalopods. This judgment was based on "ring test" data, the differential titers of which were within the experimental error of method, and generally on "plus" versus "plus-minus" reactions. In our opinion his data revealed no conclusive order of relationship among the three groups of mollusks.

Kuramoto (1933) made extensive interclass serological comparisons of representative species of pelecypods, gastropods and cephalopods. He produced antisera against the pelecypods *Ostrea gigas* Thunberg, *Corbicula japonica* Prime, *Cristaria plicata* Leach, *Cytherea meretrix* Linnaeus and *Tapes philippinarum* Adams and Reeve, the gastropods *Haliotis gigantea* Chemnitz, and *Viviparus japonicus* Martens and the cephalopods *Euprymna morsei* Verrill, and *Octopus membranaceus* Quoy. His precipitin ("ring-tests") and complement fixation data show, without question, a closer relationship between the gastropods and cephalopods than between either of these and the pelecypods. This work is singularly exceptional, in comparison to the findings of others, in that his results give strong interclass serological reactions which reciprocally verify one another.

Chestnut (1943) employed the turbidimetric analysis of precipitin reactions in his study of molluscan relationships. He produced antisera against the tissue-proteins of the pelecypods *Ostrea gigas* Thunberg and *Ostrea virginica* (Gmelin), the hemocyanins of the gastropods *Fasciolaria gigantia* Kiener, *Busycon perversum* Linnaeus, *Busycon caricum* (Gmelin), and *Aplysia protea* (Rang) and a cephalopod *Octopus* sp. The antisera against the pelecypod proteins were Class-specific, as were all but one of the antigastropod sera. A slight cross-reaction was obtained

between the anti-*Aplysia* serum and the proteins of the pelecypods. Other interclass cross-reactions were obtained with the anti-Octopus serum and the proteins from both the pelecypods and the gastropods. The gastropods were indicated to be slightly more closely related to the cephalopod than were the pelecypods.

Wilhelmi (1944) obtained reciprocal cross-reactions between antigens and antisera of the gastropod *Busycon caricum* (Gmelin) and the pelecypod *Pecten irradians* Lamarck.

Our serological data were inconclusive with respect to the establishment of a definite sequence of relationship among the four Classes of the Phylum Mollusca. The values for interclass relationships were generally low and approximately at the limit of the sensitivity of the serological methods which were employed. Multiple series of injections were given to induce the formation of powerful antisera. It was seemingly beyond the capabilities of the rabbits to produce antisera by means of which we could discriminate effectively among the cross-reacting proteins from the different Classes. Most of the proteins from species which were in the same Class as the homologous species were reactive with a given antiserum and readily distinguished from proteins from another Class. Were it not for the fact that the interclass data were chiefly at the limit of the ranges of the antisera, and close to the experimental error in the method, the negative results might otherwise have been significant as a means of distinguishing, in part, among the Classes. An inspection of the interclass data reveals several instances where the proteins of one species within a Class gave a measurable cross-reaction with an antiserum while the proteins of a second species from the same Class gave a negative result.

The pelecypod proteins gave fewer positive interclass reactions than did the proteins of the representatives of the other Classes. Moreover, most of the antisera produced against the proteins of the pelecypod species were more specific than the antisera produced against the proteins of species from other Classes. This pattern of cross-reactions for the pelecypods might have been due, in part, to the noncorrespondence of their proteins with the proteins of the species from the other Classes. The proteins of the pelecypods were in extracts obtained from homogenates of the whole bodies of organisms. Proteins from species in other Classes were the hemocyanins of the sera. The low cross-reactivity of the proteins of the amphineuran *Tonicella lineata,* which were obtained also as extracts and leachings from the bodies of the organisms, likewise might have been due to their lack of correspondence with the serum proteins of the other amphineurans. More difficult to comprehend are differences in cross-reactivity, in view of the generally aspecific nature of interclass serological reactions, of the hemocyanins from species in one Class with the antihemocyanin sera representative of another Class. The hemocyanins of the gastropod *Argobuccinum,* for example, failed to give a measurable cross-reaction with the anti-*Cryptochiton* serum, while the hemocyanins of the gastropod *Busycon* had seven per cent of measurable correspondence with the homologous reaction. The difference between the reactivities of *Argobuccinum* and *Busycon* exceeds the limits of the experimental error of the methods. Both of the antigens were collected and preserved in the same manner. Their places of origin were greatly different, but this fact should not be of importance if the same laws of inheritance and principles of systematics and evolution apply to the conservatively inherited proteins of the serum as apply to other essential characters of these

organisms. Lipoprotein-anti-lipoprotein reactions have been known (Boyden, 1936; Cumley, 1939; Wilhelmi, 1944) to account for otherwise unpredictable serological reactivity involving hemocyanins and other proteins from invertebrates. Lipoidal substances were not removed from the serum antigens which were used in our experiments. The presence of lipid materials in the serum of invertebrates might be a superficial trait, a consequence of the dietary habits of the animals, hence, under environmental control, and of trivial systematic importance in serological comparisons. Lipid materials bound to proteins as a consequence of genetically controlled syntheses are of unquestioned value in serological comparisons. The relatively aspecific nature of the fatty component of lipoproteins would provide the greatest usefulness in studies of serological correspondence among distantly related groups of animals. It was this reasoning which led us to use antigens which had not been defatted.

A critical evaluation of the interclass serological data in this paper, and in earlier reports (Erhardt, 1931; Kuramoto, 1933; Makino, 1934; Chestnut, 1943; and Wilhelmi, 1944), does not support Knight's suggestion (1952, p. 44) to merge the isopleuran (amphineuran) and anisopleuran (gastropod) mollusks into a single Class, the Gastropoda. By assuming the generally lowered interclass reactivities of the pelecypods (Table II) to be due to the lack of correspondence of their tissue-extract proteins with the hemocyanins of the sera of other groups and by recalling that most of the measurable interclass reactivities are within or near the experimental error of the method, all of the groups would stand distantly related, and approximately equally so. In this interpretation the four Classes Amphineura, Gastropoda, Pelecypoda, and Cephalopoda would be retained as commonly accepted.

Cross-reactions between serological reagents involving proteins from pelecypods, gastropods and cephalopods have been reported by Kuramoto (1933), Makino (1934), and Chestnut (1943). Each of these workers obtained results which indicated that the gastropods and cephalopods are more closely related to each other than either of them is to the pelecypods. Their data indicate, also, a closer relationship between pelecypods and gastropods, than between pelecypods and cephalopods. Our data yielded no conclusive information on the relative amounts of serological correspondence among the three groups.

Both of the anti-*Pecten* sera had reactions with the proteins of the amphineurans which were inconsistent with the other serological data in Table II. We can offer no explanation for the reactions. Subsequently-produced anti-*Pecten* sera gave no such cross-reactions with the proteins of the amphineurans. The reactions of the anti-*Macoma* serum are also unusual because of the strong cross-reaction of this serum with *Pecten* and the failure of the serum to react with the proteins of other pelecypods. These "anomalous" data are included in our results to illustrate the occasional occurrence of unverifiable serological reactions which are in all probability due to unusual responses of rabbits to the antigens being injected.

Summary

1. Serological comparisons were made with precipitins of proteins from molluscan species representative of the four Classes Amphineura, Gastropoda, Cephalopoda and Pelecypoda.

2. Interclass reactions were approximately at the limit of the sensitivity of the turbidimetric method which was employed.

3. Knight's proposal to include the amphineurans in the Class Gastropoda is not supported by the serological data. The analysis and integration of the serological data already in the literature, with the data in this work, provide additional evidence that the four groups should be retained as distinct Classes.

LITERATURE CITED

BOYDEN, ALAN, 1936. Serological study of the relationships of some common Invertebrata. *Ann. Rep. Tortugas Lab., Carnegie Inst. Washington,* **34**: 82.

BOYDEN, ALAN, AND RALPH J. DEFALCO, 1943. Report on the use of the photronreflectometer in serological comparisons. *Physiol. Zool.,* **16**: 229–241.

CHESTNUT, ALPHONSE F., 1943. Precipitin studies of certain Mollusca, with special reference to the oyster. Master's Thesis, Rutgers University Library. Unpublished.

CUMLEY, RUSSELL W., 1939. Relations among *Drosophila* species, as determined by the complement fixation reaction using ether-insoluble fractions. *J. Exp. Zool.,* **80**: 299–314.

ERHARDT, ALBERT, 1931. Die Verwandschaftsbestimmungen Mittels der Immunitätsreaktion in der Zoologie und ihr Wert für phylogenetische Untersuchungen. *Ergeb. und Fortschr. d. Zool.,* **7**: 279–377.

GALLI-VALERIO, VON B., 1916. Präzipitine und Trichotoxine für Albumine und Flimmerepithel von *Anodonta anatina* L. *Zeitschr. f. Immun.,* **24**: 311–314.

KNIGHT, J. BROOKS, 1952. Primitive fossil gastropods and their bearing on gastropod classification. *Smithsonian Miscell. Collect.,* **117**: 1–56.

KURAMOTO, MITSUGI, 1933. Über die Antigenität der Fischweichtier- und Schalfischeiweisse. *Arbeiten aus der Med. Fakultät Okayama,* **4**: 249–261.

LEONE, CHARLES A., 1949. Comparative serology of some brachyuran Crustacea and studies in hemocyanin correspondence. *Biol. Bull.,* **97**: 273–286.

LIBBY, RAYMOND L., 1938. The photronreflectometer—an instrument for the measurement of turbid systems. *J. Immun.,* **34**: 71–73.

MAKINO, KATASHI, 1934. Beobachtungen über die Immunitätsreaktionen bei Molluskenarten. *Zeitschr. f. Immun.,* **81**: 316–335.

WILHEMI, RAYMOND W., 1944. Serological relationships between the Mollusca and other invertebrates. *Biol. Bull.,* **87**: 96–105.

STUDIES ON THE SYMBIOTIC YEASTS OF TWO INSECT SPECIES, LASIODERMA SERRICORNE F. AND STEGOBIUM PANICEUM L.[1]

N. C. PANT[2] AND G. FRAENKEL[3]

Department of Zoology and Applied Entomology, Imperial College of Science and Technology, London, England

The organs and tissue cells of many insects harbor living microorganisms which seem to exert no harmful effect on their hosts. In fact, some of them have been found to bear a symbiotic relationship with the insects. It has been shown in many instances, especially in termites, blood-sucking insects and two anobiid beetles, *Lasioderma serricorne* F. and *Stegobium* (*Sitodrepa*) *paniceum* L., that symbionts can play an important part in the nutrition of their host (Fraenkel, 1952; Buchner, 1953).

The mycetom which houses the yeast-like symbionts of *Stegobium* was first described by Karawaiew (1899), and the true nature of the symbionts first recognized by Escherisch (1900). Later Buchner (1912), Heitz (1927), Breitsprecher (1928), Koch (1933), Fraenkel and Blewett (1943), Blewett and Fraenkel (1944) and Pant and Fraenkel (1950) added to our knowledge of the relationship of the symbionts to their insect hosts in *Lasioderma* and *Stegobium*. Koch (1933) recognized that the symbionts of *Stegobium* exerted a function in the nutrition of the host which was similar to that of yeast. Blewett and Fraenkel (1944) showed that the symbionts were sources of many of the vitamins of the B-complex for their hosts. This present study continues and extends the previous work by Blewett and Fraenkel and is also concerned with the culture of the yeasts outside the body and the effect of transplanting them into the foreign host. Some of the results have already been briefly reported (Pant and Fraenkel, 1950).

Materials and Methods

Cultures of *Lasioderma serricorne* and *Stegobium paniceum* were maintained on wholemeal flour and wheat bran with the addition of 5% dried debittered brewers yeast. The cultures had to be covered with tightly fitting wire gauze tops because of the tendency of the adult beetles to cut through muslin covers. In order to obtain a large number of eggs, adult beetles were maintained on a small quantity of white flour plus 5% yeast. Eggs laid in this medium were recovered by sifting through a 60-mesh sieve.

The basic diet was the same as that described previously for *Tenebrio molitor* (Fraenkel *et al.*, 1950) and consisted of 20 parts casein, 80 parts glucose, 1 part cholesterol and 2 parts McCollum's salt mixture no. 185. To this mixture the

[1] This study represents part of a thesis accepted by the University of London for the Ph.D. degree of N. C. Pant.

[2] Present address: Department of Zoology, University of Delhi, India.

[3] Present address: Department of Entomology, University of Illinois, Urbana, Illinois.

following vitamins of the B-complex were added (expressed as μg. per gram of the dry diet) : thiamin 25, riboflavin 12.5, nicotinic acid 25, pyridoxin 12.5, pantothenic acid 25, choline chloride 500, inositol 250, folic acid 2.5 and biotin 0.1. In some cases the water-insoluble residue of yeast was used in the place of biotin (Fraenkel and Blewett, 1943).

The tests were carried out in 2 × 1 inch shell vials closed by one-holed corks with muslin tops. Ten newly hatched larvae were added to each tube and each test was performed in duplicate. Each tube contained two grams of food. All tests were carried out in a constant-temperature-humidity chamber of 27° C. and 70% relative humidity.

FIGURE 1. Intestine of the adult *Lasioderma serricorne*, showing the mycetoms at the junction of fore- and midgut.

For the study of the symbionts *in situ*, sections were cut and stained with Delafield's hematoxylin and eosin or orange G. Smears of mycetomic tissue were stained with Wright's stain or were smeared on to the slide with a drop of India ink. The latter method proved very suitable for the study of the shape of the cells.

For the graphic representation of results, the total number of adults formed was plotted against time. Both *Lasioderma* and *Stegobium* spin a cocoon previous to pupation. Most of these cocoons are fixed on to the glass wall of the vials leaving open a window to the inside of the cocoon through which the insects can be observed. Thus the time of pupation and emergence of the adults can be accurately determined by frequent inspections.

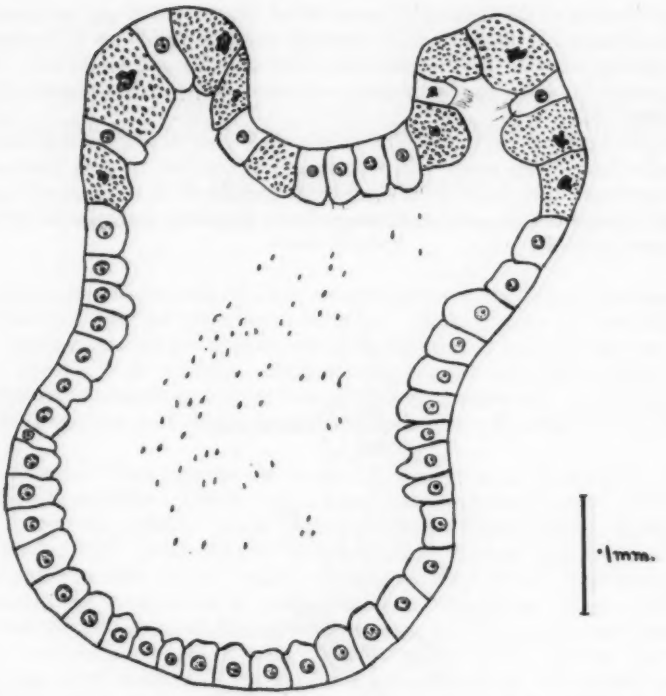


FIGURE 2. Transverse section through two lobes of the mycetom of the *Stegobium paniceum* larva, showing location of symbionts in the mycetom and lumen of the intestine.

OBSERVATIONS AND EXPERIMENTS

1. Mycetoms and symbionts

The mycetoms of *Lasioderma serricorne* are similar to those of *Stegobium paniceum* which have already been described several times (Karawaiew, 1899; Buchner, 1912; Heitz, 1927; Breitsprecher, 1928). However in *Lasioderma* they consist of 6 lobes or protrusions situated at the junction of fore and midgut which are evaginations of the wall of the midgut, and which are in continuation with the lumen of the gut (Fig. 1). As in *Stegobium*, they consist of large cells containing symbionts and irregular shaped nuclei. The other cells of the epithelium are without microorganisms and have round nuclei and fringed borders (Fig. 2).

In newly hatched larvae the mycetoms are not externally differentiated. It is after a period of 5–7 days that the mycetoms acquire their characteristic lobed shape. In the prepupae the mycetomes become reduced in size and are no longer prominent. They are further reduced in the pupae, but are fully developed in the adults, where each lobe is outwardly subdivided into three sub-lobes. In *Stegobium*, which has a four-lobed mycetom, the shape of the organ changes markedly in the adult, by the development of six tubular appendages which arise from each lobe.

The symbionts of *Stegobium* were first identified by Escherisch (1900) as organisms which look like yeasts and multiply by budding. They differ markedly in size and shape in the two species (Figs. 3 and 4). In *Lasioderma* the cells are broadly oval, 2 to 3.5 μ wide and 2 to 4.5 μ long, with only one bud attached to a cell, except for the pupa, where two buds per cell are common. The buds are apical and the point of attachment is broad. No mycelium or spores were ever observed. In the adults the symbionts are slightly smaller and budding is less common. In *Stegobium* the yeasts are elongate, pear-shaped, and pointed at one end to which a single bud may be attached. The point of attachment is very narrow. The size varies from 1.5 to 3.5 μ width and 3 to 6 μ length. These differences in size, shape and budding are so characteristic that the two types can be readily discerned.

2. Cultivation of the symbionts

Escherisch (1900) claimed to have cultivated *Stegobium* but Heitz (1927) and Breitsprecher (1928) failed in subsequent attempts. We have successfully cultivated the yeasts of both species in Hansen's solution (peptone 1.0 gm., glucose 5 gm., potassium dihydrogen phosphate 0.3 gm., magnesium sulfate 0.3 gm., and water 100 ml.). The larvae were sterilized by submerging them in 5% chloramine in 70% alcohol for two minutes. They were then washed with sterilized water, and the mycetom dissected out under sterile conditions and transferred into the culture medium. In the case of *Lasioderma,* inoculated medium was kept at 29° C. for 15 to 20 days after which it turned turbid, with the cells settling down

FIG. 3

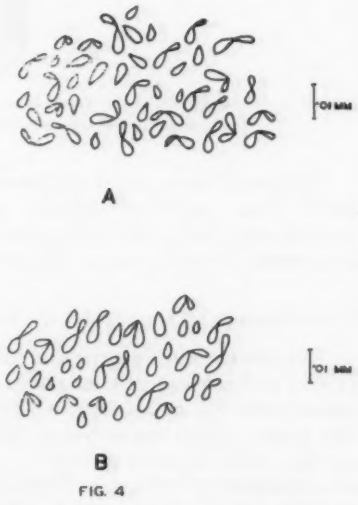


FIG. 4

FIGURE 3. The yeast-like symbionts of *Lasioderma serricorne.* A. In the larva. B. In the pupa.

FIGURE 4. The yeast-like symbionts of *Stegobium paniceum.* A. In the larva. B. Cultivated symbionts.

at the bottom. The suspension was then inoculated on to an agar medium where colonies were formed (Fig. 5). The incubation period for the yeasts from *Stegobium* was longer, up to 35 days, and growth was poor compared with that of *Lasioderma* yeasts. No colonies were obtained on the agar medium.

The cultivated yeasts showed the same morphological characteristics as those *in situ* (Figs. 3 and 4). But in very old cultures chains of yeast sometimes developed. *Lasioderma* symbionts could be grown in such profusion that it was possible to conduct feeding trials with them. For inoculation of media mycetoms of larvae, pupae and adults were used with success. Eggs were also employed but given up, owing to the fact that the outer surface, which contained the yeasts, could not be sterilized.

FIGURE 5. A. Cultures of the symbionts of *Lasioderma* on an agar medium. B. Culture of the same symbionts after implantation into *Stegobium* larvae.

The yeasts from either species have never been accurately identified taxonomically. They were provisionally placed by Buchner (1930) under the genus *Saccharomyces* and named *S. anobii*. However they differ from *S. cervicae* in that they cannot ferment glucose with development of carbon dioxide.

3. Elimination of yeasts from the insects, and reimplantation

The insects were deprived of their yeast by a method first described by Koch (1933) and subsequently employed by Blewett and Fraenkel (1944). Eggs were treated with 5% chloramine in 70% alcohol for one minute at room temperature. The yeasts, which are normally transmitted by smearing on the outer surface of the egg, while it passes through the common oviduct, are hereby killed. The eggs are subsequently washed in distilled water. The larvae which hatched from these eggs did not contain symbionts, although the mycetoms developed normally. The offspring from eggs treated in this way will never acquire symbionts, unless reinfected.

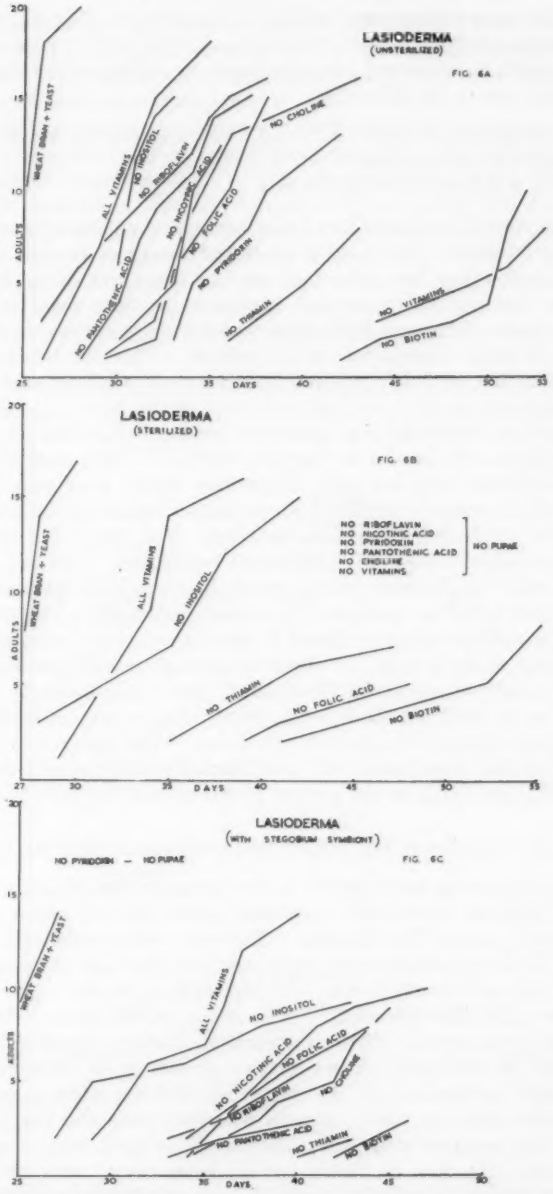


FIGURE 6. Growth of the larvae of *Lasioderma serricorne* on artificial diets in the presence and absence of certain vitamins of the B-complex. A. Normal larvae. B. Larvae without symbionts. C. Larvae which contain the symbionts of *Stegobium*.

When sterile eggs were treated with a suspension of cultivated yeasts the ensuing larvae became reinfected with symbionts. "Sterilized" larvae of *Lasioderma* or *Stegobium* could be reinfected with symbionts by adding to the flour, which was otherwise sterile, one of the following:

1. Culture of cultivated yeast (done only with *Lasioderma* yeasts);
2. 0.1 g. faeces of normal insects in 2 g. of flour;
3. flour from a culture of normal insects.

Sterilized larvae of *Lasioderma* on a diet containing cultivated *Lasioderma* yeast readily became infected. Food which contained faeces of normal larvae did not affect *all* sterilized larvae, but, after two months, *some* individuals of *Lasioderma* and *Stegobium* were found to contain symbionts in their mycetoms. Flour on which normal insects had been feeding previously had no effect on the sterility of the mycetoms of either *Lasioderma* or *Stegobium*. This can be explained by the much smaller amount of faeces present in this sample than the one where faeces had been added.

It was, therefore, concluded that larvae are readily infested by way of mouth, by admixing a culture of the symbiotic yeasts to the food. This method could only be used with *Lasioderma* and not with *Stegobium* whose symbionts did not grow sufficiently well in culture. Sterilized *Stegobium* larvae could, however, be readily infested with the cultivated yeast of *Lasioderma*. The yeasts in the mycetoms retained the characteristic shape of *Lasioderma* symbionts. Cultures were successfully obtained from *Lasioderma* yeasts grown in *Stegobium* mycetoms under circumstances which failed to produce *Stegobium* symbionts. The adults of *Stegobium* with *Lasioderma* yeast produced a generation which contained the typical *Lasioderma* yeast, and this could be observed through several generations.

Stegobium symbionts could be "inoculated" into *Lasioderma* by smearing cultures of them on to sterilized *Lasioderma* eggs. The mycetoms became filled with yeast of the typical shape of *Stegobium* symbionts. The mycetomic tissues of such larvae of *Lasioderma* were inoculated into Hansen's medium and the growth was found to be poor, analogous to the growth of the normal *Stegobium* symbionts.

4. Physiology of Lasioderma and Stegobium symbionts in their normal host

Larvae of *Lasioderma* were grown in the presence and absence of the normal symbionts on artificial diets which contained either the full vitamin complement, or were lacking in one of the vitamins. The tests were performed in two series, one in which the diet contained the water-insoluble fraction of yeast (which contains biotin), and one in which this was replaced by biotin. The results of the two tests were essentially identical. The normal larvae grew relatively well in diets which did not contain riboflavin, inositol, choline chloride, nicotinic acid, pantothenic acid or folic acid. Diets without pyridoxin or thiamin were slightly inferior although the majority of the larvae reached the adult stage. In the absence of insoluble yeast or biotin, growth was very slow and the mortality high. The sterilized larvae grew well in the absence of inositol, very poorly in the absence of thiamin, folic acid or biotin (or insoluble yeast) and entirely failed to grow in the absence of riboflavin, nicotinic acid, pyridoxin, pantothenic acid and choline chloride (Fig. 6).

When sterilized larvae had been reinfested with their own yeast by feeding them on yeast cultures, they grew about as well in the absence of single vitamins as did normal larvae. This clearly indicates that growing the yeast cells in pure culture did not change them physiologically.

A comparison was also made between growth of *Stegobium* in the presence and absence of the symbionts on diets which contained either the full vitamin complement, or had a single vitamin omitted. With the symbionts present the larvae grew very well in the absence of inositol; omission of pyridoxin, riboflavin, nicotinic acid, folic acid or pantothenic acid delayed growth very slightly, but little or no growth took place in the absence of biotin or thiamin respectively. With the symbionts absent, no growth occurred in the absence of thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, biotin or folic acid. Growth was markedly delayed in the absence of choline chloride and unaffected in the absence of inositol (Fig. 7).

In general, the effect of symbionts in diets lacking in certain vitamins was similar in *Lasioderma* and *Stegobium*. Certain differences which emerged from these

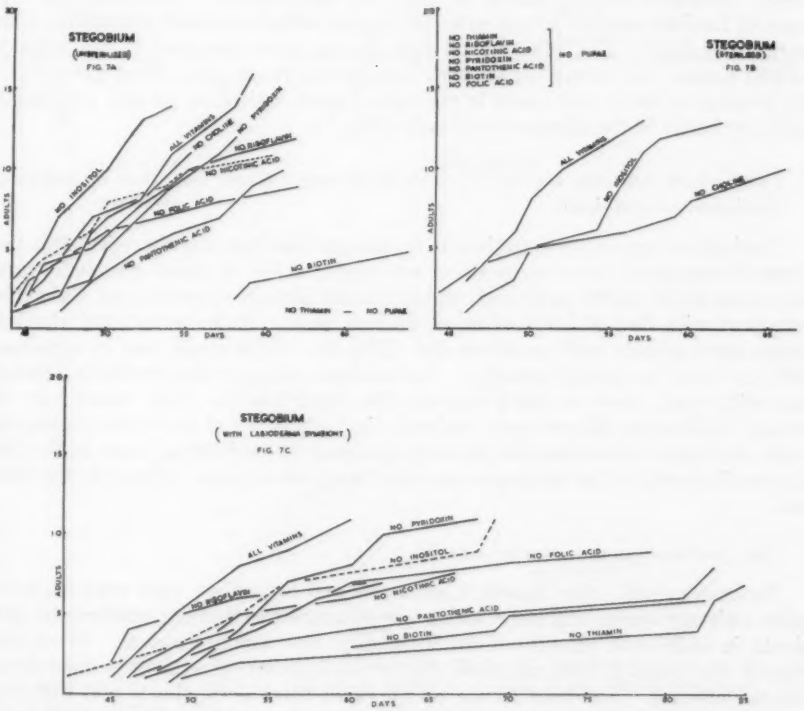


FIGURE 7. Growth of the larvae of *Stegobium paniceum* in the presence and absence of certain vitamins of the B-complex. A. Normal larvae. B. Larvae without symbionts. C. Larvae which contain the symbionts of *Lasioderma*.

tests appear to be connected rather with the requirements of the insects than the properties of symbionts. The normal *Lasioderma* grew slowly in the absence of thiamin, but so also did the larva after removal of the symbionts. Neither the normal nor sterilized *Stegobium* grew in the absence of thiamin. It seems, therefore, that neither *Lasioderma* nor *Stegobium* symbionts supply sufficient quantities of thiamin. The sterilized *Lasioderma* failed to grow in the absence of choline chloride, while choline was not required by the sterilized *Stegobium*.

5. The effect of the symbionts of Lasioderma and Stegobium in the abnormal host

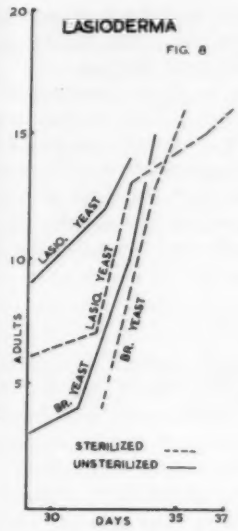
Sterilized *Lasioderma* was infected with the symbionts of *Stegobium* by the method of smearing a culture of *Stegobium* yeasts on the sterilized eggs. The resulting larvae were then submitted to the series of diets, which were lacking in certain factors, as has been described before. Growth of these larvae was better than that of sterilized larvae but inferior to that of normal larvae (Fig. 6). In most instances growth was delayed and the mortality increased. However, in only one case, in the absence of pyridoxin, did the *Lasioderma* larvae with *Stegobium* symbionts entirely fail to develop. In the converse experiment cultivated yeast of *Lasioderma* was mixed with the food on which sterilized *Stegobium* larvae were maintained. The offspring of these larvae were submitted to the tests described above. *Stegobium* larvae with *Lasioderma* yeasts grew about as well as in the presence of their own yeasts in the case of most individual vitamin deficiencies, and even better in the absence of thiamin (Fig. 7).

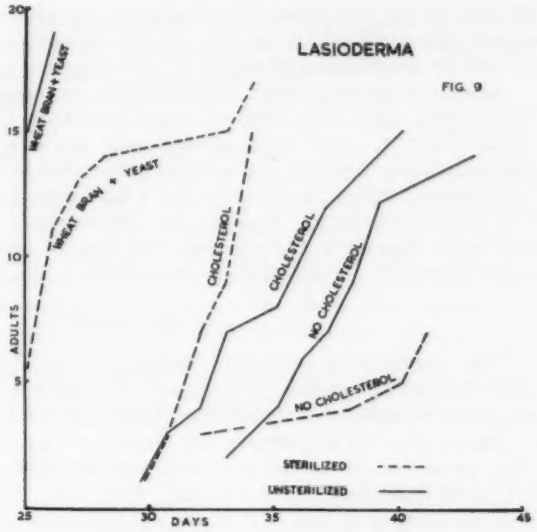
6. Comparison between the effect of dried brewers yeast and that of cultivated Lasioderma symbionts

Lasioderma yeasts were cultivated on an agar medium and the yeast cells then carefully separated from the medium and dried; 2.5% of dried symbiotic yeasts were then added to the basic diet, which did not contain vitamins, and their effect compared with that of 2.5% of dried brewers yeast. Both normal and sterilized larvae grew equally well on either diet (Fig. 8). This result was in agreement with the tests previously reported. *Lasioderma,* owing to its symbiotic relationship with yeast, does not need vitamins like other insects. The reason why the normal *Lasioderma* did not grow optimally in the absence of pyridoxin or thiamin, while sterilized *Lasioderma* did so in the presence of *Lasioderma* yeast in the diet, can be attributed to the far larger amounts of symbiotic yeast present in the latter case.

7. The symbionts as a source of sterols

By analogy with other insects, *Lasioderma* and *Stegobium* were expected to require a dietary source of a sterol and it was of interest to find out whether the symbionts, in addition to vitamins of the B-complex, also supplied sterols. When cholesterol was omitted from the basic diet of *Lasioderma,* growth of normal larvae was not affected. Sterilized larvae, on the other hand, grew slowly and with high mortality in the absence of cholesterol. When cholesterol was added the efficiency of the diet was at once restored (Fig. 9). It is evident that a sterol deficiency does not arise as long as symbionts are present in the insect. In another test the





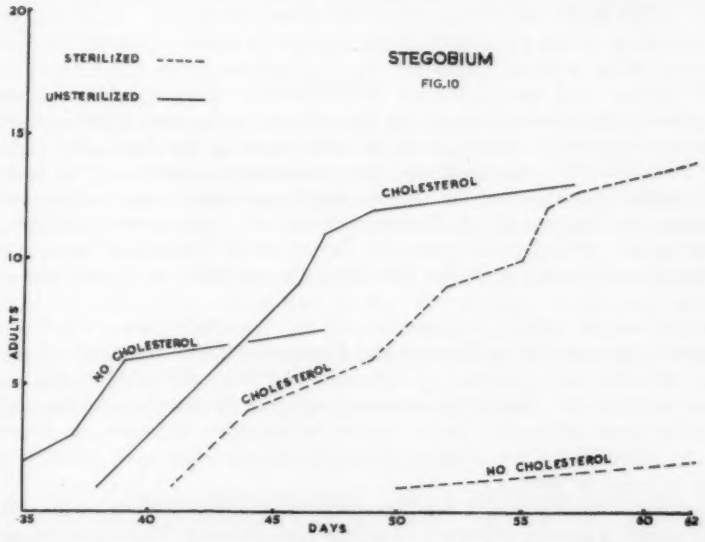


FIGURE 8. Growth of normal and symbiont-free larvae of *Lasioderma* in the presence of dried symbionts or dried yeast as sources of vitamins. L.Y. = Dried *Lasioderma* yeast. Br. Yeast = Dried brewers yeast.

FIGURE 9. Growth of normal and symbiont-free larvae of *Lasioderma* in the presence and absence of cholesterol.

FIGURE 10. Growth of normal and symbiont-free larvae of *Stegobium* in the presence and absence of cholesterol.

presence of the insoluble yeast fraction in the diet resulted in normal growth in normal and sterilized larvae alike, in the absence of cholesterol. This can be attributed to the presence of sterols, largely ergosterol, in the yeast fraction.

Results regarding sterol requirements, corresponding to those reported for *Lasioderma,* were also obtained with *Stegobium* (Fig. 10). Both normal and sterilized larvae grew about equally well in diets which contained cholesterol. Without cholesterol there was a marked difference in both types of larvae. Normal larvae grew rapidly, though there was a high mortality, but there was little growth in the sterilized larvae. It seems that the symbionts of *Lasioderma* supply more cholesterol than those of *Stegobium,* also that the absence of sterol from the diet is more critical for *Stegobium* than *Lasioderma.*

Discussion

The results of the present investigation restate and largely confirm the conclusions drawn in an earlier investigation (Blewett and Fraenkel, 1944), that the intracellular symbionts of *Lasioderma* and *Stegobium* have a nutritional effect similar to that of yeast in a synthetic diet, namely to supply vitamins of the B-complex. They go beyond the earlier results in that folic acid and biotin have been added to the list of individual vitamins tested. Folic acid, like many of the other vitamins, does not seem to be necessary in the diet when the larvae have their ordinary complement of symbionts, but becomes a critical requirement in the absence of the symbionts. With biotin the results were not quite conclusive. Neither *Lasioderma* nor *Stegobium* larvae grow well in the absence of biotin, even in the presence of symbionts, while without symbionts, the *Lasioderma* larvae gave about the same growth pattern and the *Stegobium* larvae entirely failed to grow. There were some minor discrepancies between the present and earlier investigation which might be due to a variety of factors, such as differences in the basic diet (addition of biotin and folic acid), the casein, and the environmental factors, or the insect stock. In the earlier investigation, but not in the present one, choline was a critical requirement for the normal *Lasioderma* larva. On the former occasion choline chloride was a critical requirement for the sterilized *Stegobium* larva, while now the presence or absence of choline had little effect on both the normal and sterilized larva.

Results on the vitamin requirements of the *Stegobium* larva, which agree well with those given earlier by Blewett and Fraenkel (1944) and again in this publication, were recently reported by Lemonde and Bernard (1953), who succeeded in growing them on a diet which contained only two B-vitamins, thiamin and biotin. The larvae grew noticeably slower than on wheat flour; however, no improvement could be achieved by the addition of riboflavin, nicotinic acid, pantothenic acid, choline chloride or folic acid.

In general, the impression has been obtained that the symbiotic yeasts of *Lasioderma* exhibit a greater efficiency of action than those of *Stegobium. Lasioderma* yeasts in the *Stegobium* host have about the same effect as, and with regard to thiamin, an even better effect than, the *Stegobium* yeasts. *Stegobium* yeasts are less efficient in *Lasioderma* than the normal symbiont. This could be, however, attributed to the same unexplained phenomenon that *Lasioderma* yeasts are easier to culture than *Stegobium* yeasts. All these phenomena can be reduced to one

common factor, namely the greater synthetic faculties of *Lasioderma* yeast. It would be interesting to compare vitamin contents of the two species of yeast, and also with those of known varieties of bakers or brewers yeast.

Finally the question remains to be discussed as to how the intracellular symbionts exert their nutritional effect. It has been reported earlier that toward the end of larval life, in the prepupa, the mycetoms gradually are reduced in size. Breitsprecher (1928) described that during this period large masses of yeasts, even whole mycetocytes, enter the lumen of the intestine and are eliminated with the faeces without digestion taking place. In our experience, yeast cells are liberated from the mycetoms all through the growing period of the larva, and only relatively few of these cells are found in the faeces (Fig. 2). It is therefore possible that the host acquires vitamins by digesting the symbionts. The alternative, difficult to prove, would be a diffusion of vitamins from the symbionts into the cytoplasm of the mycetocytes and hence to the hemolymph of the host. This process would differ little from the well known phenomenon of an enrichment of any microbial culture medium by the synthesis in, and diffusion from, the cultured microbe.

Acknowledgment

One of us (N. C. Pant) wishes to acknowledge a grant made by the Government of India which made this investigation possible, and to thank also Dr. O. W. Richards, Imperial College, London, for many helpful suggestions, and Miss Mary Coles for technical help.

Summary

1. The shape and appearance of the yeast-like symbionts of *Lasioderma serricorne* F. and *Stegobium paniceum* L. have been described. The symbionts have been cultured in Hansen's solutions and on an agar medium.

2. The larvae of both species are relatively little affected by the absence of certain vitamins of the B-complex. After elimination of the symbionts these vitamins become absolutely necessary.

3. Sterilized larvae of either species have been successfully re-infected with the symbionts of the other host, which then retain their peculiar shape and mode of action.

4. Cultured symbionts exert the same growth promoting effect as dried brewers yeast when added to the food.

5. The symbionts are also sources of sterols for their hosts.

LITERATURE CITED

Blewett, M., and G. Fraenkel, 1944. Intracellular symbiosis and vitamin requirements of two insects, *Lasioderma serricorne* and *Sitodrepa panicea*. *Proc. Roy. Soc. London, Ser. B*, **132**: 212–221.

Breitsprecher, E., 1928. Beiträge zur Kenntnis der Anobiidensymbiose. *Zeitschr. Morph. Ökol. Tiere*, **11**: 495–538.

Buchner, P., 1912. Studien an intracellulären Symbionten. *Arch. Protistenk.*, **26**: 1–116.

Buchner, P., 1930. Tier und Pflanze in Symbiose. Gebr. Bornträger, Berlin.

Buchner, P., 1953. Endosymbiose der Tiere mit pflanzlichen Mikroorganismen. Birkhäuser, Basel.

ESCHERISCH, K., 1900. Über das regelmässige Vorkomman von Sprosspilzen in dem Darmepithel eines Käfers. *Biol. Zentr.*, **20**: 350–358.

FRAENKEL, G., 1952. The role of symbionts as sources of vitamins and growth factors for their insect hosts. *Tijdschr. Entomologie*, **95**: 183–195.

FRAENKEL, G., AND M. BLEWETT, 1943. The vitamin B-Complex requirements of several insects. *Biochem. J.*, **37**: 686–692.

FRAENKEL, G., M. BLEWETT AND M. COLES, 1950. The nutrition of the mealworm *Tenebrio molitor*. *Physiol. Zool.*, **23**: 92–108.

HEITZ, E., 1927. Über intrazelluläre Symbiose bei holzfressenden Käferlarven. *Zeitschr. Morph. Ökol. Tiere*, **7**: 279–305.

KARAWAIEW, W., 1899. Über Anatomie und Metamorphosis des Darmkanals der Larve von *Anobium paniceum*. *Biol. Zentr.*, **19**: 122–130, 161–171, 196-220.

KOCH, A., 1933. Über das Verhalten symbiontenfreier Sitodrepa Larven. *Biol. Zentr.*, **53**: 199–203.

LEMONDE, A., AND R. BERNARD, 1953. Aspects nutritives des larves de *Stegobium paniceum* L. (Anobiidae) et d' *Oryzaephilus surinamensis* L. (Cucujidae). *Naturaliste Canad.*, **80**: 125–142.

PANT, N. C., AND G. FRAENKEL, 1950. The function of the symbiotic yeasts of two insect species, *Lasioderma serricorne* F. and *Stegobium* (Sitodrepa) *paniceum* L. *Science*, **112**: 498–500.

THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. I. MOLTING AND GROWTH IN LABORATORY-MAINTAINED INDIVIDUALS[1]

DOROTHY F. TRAVIS[2]

Bermuda Biological Station for Research[3]

Since the summer of 1949, the author has become deeply interested in the physiology of molting and growth in the Bermuda spiny lobster, *Panulirus argus* Latreille. Investigations have been concerned with normal morphological and physiological changes related to molting and growth in this animal. Interest has centered around the changes which occur in the skeleton, tissues, blood, and urine of normal animals during the molting cycle.

The present paper, the first of a series, is concerned with the spectacular process of molting, the mechanics by which it is achieved, some physical factors influencing molting and growth, and growth determined by weight and length in laboratory-maintained spiny lobsters.

Materials and Methods

Male and female spiny lobsters, ranging in carapace length from 20–92 mm., were generously supplied by the Bermuda Biological Station and Dr. W. H. Sutcliffe, who furnished the author with all immature forms from his catches of 1952. Only twenty-seven of approximately 500 animals supplied were of the carapace length range of 90–92 mm. These animals were included in the data for Figure 11. No further use was made of these animals following the first laboratory molt because Sutcliffe (1952, 1953) has pointed out that sexual maturity in females is attained some time after the carapace length reaches 90 mm. Information was almost exclusively limited, therefore, to animals of 20–89 mm. carapace length. All animals, when brought into the laboratory, were numbered with a lead or plastic tag, wired to the base of an antenna. Their number, sex, weight and carapace length were recorded. The animals were then placed in concrete tanks of running sea water ($22' \times 3' \times 1\frac{1}{2}'$). By partitioning portions of the sea water tanks with plastic screens, premolt animals could be isolated from the others. At the time of ecdysis animals were placed in individual aquaria or in partitioned portions of the tanks. Following ecdysis, animals were allowed to harden before being placed in partitioned

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[2] Present address: Department of Zoology, University of New Hampshire, Durham, New Hampshire.

[3] Contribution No. 214.

portions of tanks in different size groups. The laboratory history of each animal was followed closely throughout the period of observation by the method described. The method of designating stages in the molting cycle by letters A, B, C, D (Drach, 1939), as well as the method by which the molting cycle is divided into stages by daily intervals, was used.

The weights of animals throughout the molting cycle were obtained by carefully placing them on paper towels and drying them as thoroughly as possible. The animals were then rolled in a piece of nylon screen which restricted their movements while being weighed on a triple beam balance, accurate to 0.3 gm.

Length measurements were made with a vernier caliper from the mid-dorsal anterior point between the two rostral spines to the most posterior mid-dorsal point of the carapace.

In all cases where the standard error of the arithmetical mean was calculated, the method of Fisher (1948) for small samples was used.

With few exceptions, all animals were fed every other day. Their laboratory diet consisted of fish fry, anchovies, small pieces of larger fish, molluscs, and ground beef when the bait supply became low during the winter months.

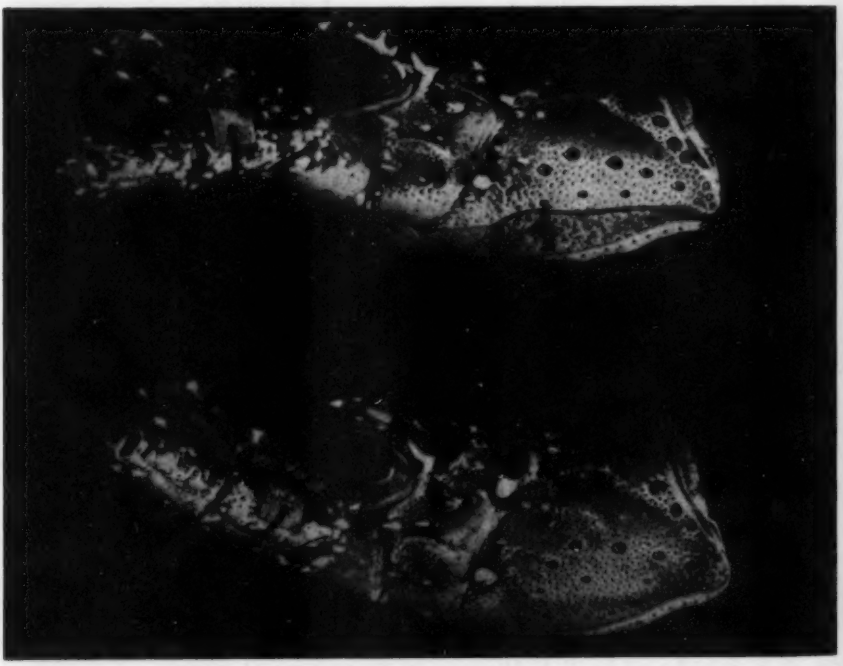

FIGURE 1. The shed (A) and hard carapace (B) of *Panulirus argus*. In the exuvium, the resorptive or ecdysal line (1) and the area ventral to this, in which somewhat less resorption occurs (2), will be noted. Neither the ecdysial line nor the softened area ventral to the line will be noted in the hard carapace, which was removed from an animal in Stage C of the molting cycle.

Observations and Discussion

In *Panulirus argus,* a premolt animal can easily be detected by the appearance of a resorptive or ecdysial line along the branchiostegites (Fig. 1). This line becomes clearly evident 3–4 days preceding ecdysis in the summer months. Beneath this resorptive line appreciable softening (20% resorption—Travis, 1951a) of the whole ventral area occurs. Premolt animals can thus be detected by feeling this softened area even before the resorptive or ecdysial line is clearly evident. In the winter months, premolt animals can be detected by touch as early as 14–21 days

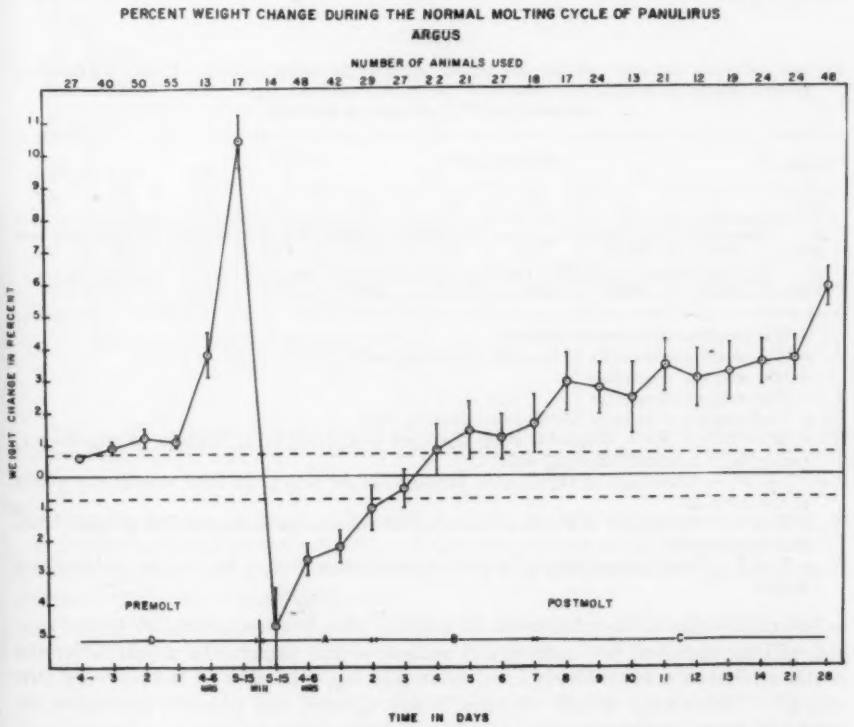
PERCENT WEIGHT CHANGE DURING THE NORMAL MOLTING CYCLE OF PANULIRUS ARGUS
NUMBER OF ANIMALS USED
27 40 50 55 13 17 14 48 42 29 27 22 21 27 18 17 24 13 21 12 19 24 24 48
WEIGHT CHANGE IN PERCENT
PREMOLT
POSTMOLT
A
B
C
4-6 HRS
5-15 MIN
5-15 MIN
4-6 HRS
TIME IN DAYS

FIGURE 2A. The per cent weight change during the normal molting cycle of *Panulirus argus.* The number of animals used per point on the graph is indicated along the top ordinate. All points represent the mean arithmetical values obtained from the number of animals specified. The vertical lines represent plus or minus the standard error of the mean. The solid horizontal line marked by (o) on the graph, represents the base weight (intermolt weight) upon which per cent changes were calculated. Dashes above and below the solid horizontal line represent the standard daily variation in weight of 24 animals. This daily variation was observed over a 7-day interval during the intermolt period, a time at which the weight of the animals is stable. The vertical line at the bottom of the graph separates the premolt and postmolt period. The bottom ordinate represents the time in days preceding and following molting. The solid horizontal lines parallel to the bottom ordinate and broken by the letters D, A, B, and C designate the four major stages in the molting cycle as utilized by Drach, 1939.

preceding ecdysis. The lengthening of the premolt period is brought about by lower winter temperatures, which markedly slow down physiological activities associated with the molting process.

One of the most manifest external changes noted a few hours before ecdysis in *Panulirus* is the swollen condition of the intersegmental membranes separating the cephalothorax and abdomen. Due to resorption from the resorptive or ecdysial line, along the branchiostegites, and resorption from an articulating condyle connecting the branchial chamber to the posterior edge of the branchiostegites, lateral expansion of the soft body beneath the old skeleton is allowed. Such swelling and lateral expansion of the thorax immediately preceding ecdysis were first thought

TABLE I

The per cent water absorbed and per cent weight increases preceding molting. Values are based on normal changes in weights associated with ecdysis in Panulirus argus. The abbreviations of Guyselman (1953) are used in this table

Number of animals used	Weight in grams						E as % of W_1	W_2 as % of I	W_1 as % of I
	I	W_1	E	E_1	W_2	W_2			
3	519.2–539.4	571.7–596.4	92.7–124.0	50.5–55.8	511.2–544.0	40.3–70.0	15.6–21.7	7.5–13.5	10.1–11.6
Avg.	528.5	584.4	111.9	53.0	524.1	51.9	19.2	9.8	10.9
3	623.3–668.9	663.5–730.1	103.3–140.5	56.0–65.2	620.0–663.5	30.8–73.9	15.6–19.2	4.7–11.0	6.5–9.3
Avg.	647.9	704.2	124.5	60.6	636.5	56.9	17.6	8.8	8.3

I = Wet weights of intermolt animals
W_1 = Wet weights of animals 15 minutes preceding molt
E = Wet weights of exuviae
E_1 = Dry weights of exuviae
W_2 = Wet weights of animals 15 minutes following molt
$W_2 = W_2 - (W_1 - E)$ = Weight of water absorbed in a period taken between 15 minutes preceding to 15 minutes following molt
E as % of W_1 = The weight of the exuviae, expressed as per cent of the total premolt wet weight of the animals
W_2 as % of I = The weight of the water absorbed expressed as percentage of the previous intermolt wet weight
W_1 as % of I = Total premolt weight increase expressed as per cent of the previous intermolt wet weight

to be entirely due to the absorption of water. This has not proved to be the case and will be discussed in a subsequent section of this paper. In a search for the causal mechanisms immediately involved in molting, a number of factors were considered. These were weight changes, water uptake, and possible structures involved in water retention.

Weight changes

In *Panulirus,* marked changes in body weight are observed immediately preceding ecdysis and for a four-week period following it. These wet weight changes, expressed as per cent of the previous intermolt weights, were followed throughout the normal molting cycle of the spiny lobster and are shown in Figure 2A. It will be observed in the 80–89 mm. carapace length animals, represented in this graph, that little change in weight is noted in the pre-ecdysial period until the day preceding ecdysis. At this time, the first significant weight increase is observed a

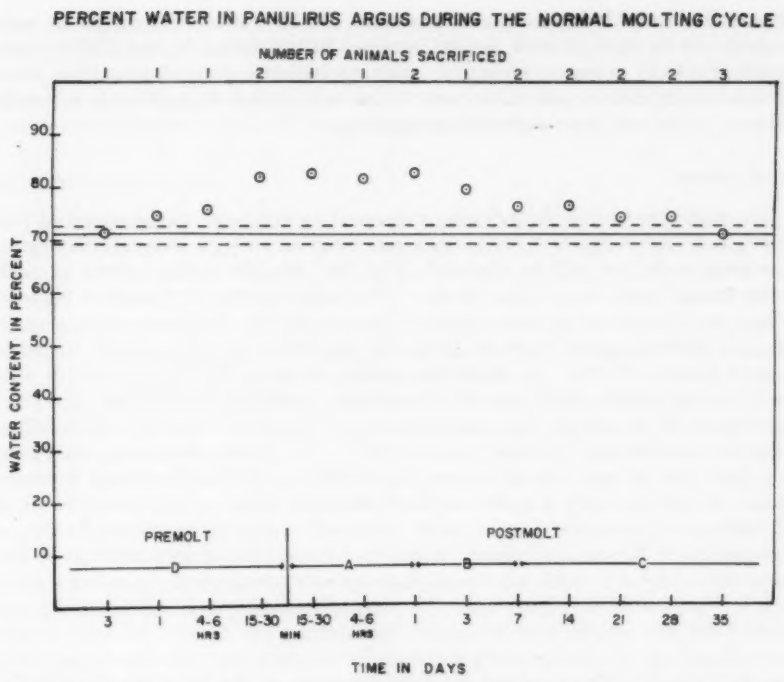

FIGURE 2B. Changes in water content of *Panulirus argus* during the normal molting cycle. Dashes above and below the horizontal line indicate plus or minus variations from the intermolt mean.

few hours preceding molting. The weight of the animals continues to increase to a peak premolt value of around 10%. This peak premolt value is attained 5 to 15 minutes preceding ecdysis. The marked increase in weight attained at this time is primarily the result of water absorbed.

During the early post-ecdysial period (15 minutes) a striking weight loss is observed. This weight loss of almost 16% is primarily due to the loss of the old exuviae, which constitute from 15.6 to 21.7% of the total premolt wet weight of the animal (Table I). Within 4 to 6 hours, however, the weight begins to rise. This continues at a rather steady pace, reaches the intermolt value two to four days following molt, significantly rises above this level at eight days, tends to level off and reaches the new intermolt value between 28 and 35 days.

Water uptake

It was mentioned earlier that the peak premolt weight increase of 10% was the result primarily of water absorbed. This is indicated in Table I. Here the weight of the water absorbed preceding molt is determined (Drach, 1939; Guyselman, 1953) and expressed as per cent of the previous intermolt wet weight. The premolt weight increase, expressed as per cent of the previous intermolt wet weight

determined as indicated in the previous section, is also listed. The weight of water absorbed and the total premolt weight increase, both obtained by two different procedures (Table I), compare favorably. Weight increases preceding molting, therefore, are entirely due to water absorbed. This is evident both in animals within the 500 gram range and those in the 600 gram range.

Water content

The water content of the animals, expressed as per cent, was determined from the wet and dry weights of individuals sacrificed at various intervals throughout the molting cycle. It will be observed (Fig. 2B) that the water content of an intermolt animal (late stage C) is 71.0%. The water content of *Panulirus* begins to increase the day preceding molt (74.7%), rises to 81.3% fifteen minutes preceding molt, and reaches a peak value of 82.4% one day following molt, 11.4% above the intermolt value of 71.0%. It should be pointed out here that the increase in water content of individuals sacrificed 15–30 minutes preceding molt (Fig. 2B) is in agreement with the weight increase observed in Figure 2A and the calculated per cent water absorbed in this same period (Table I). Since presumably all mineral and organic resorption is completed in the old skeleton at 15–30 minutes preceding ecdysis, the wet and dry weights of these skeletons must be subtracted from the total wet and dry weights of the animals with their old skeletons, to obtain the true water content of the animals alone. If this is not done the weight increase of 10% is observed (Fig. 2A) with no rise of corresponding magnitude in water content, which is represented in Figure 2B. Because all animals used in Figure 2B were sacrificed for dry weight determinations, the average per cent of the total weights of the animals due to exuviae, both wet and dry weights, was calculated from values given in Table I. These values were then applied to the two animals sacrificed 15–30 minutes preceding molt (Fig. 2B) to obtain the presumed wet and dry weights of the old skeleton of these animals. The water content of these animals, without their old skeletons, was then calculated. From three days following molt the water content slowly declines, but does not return to its intermolt range of 71.0% before 21 to 28 days. It might be mentioned that at 28 days the animals have fully calcified their new skeletons (the principal layer and the membranous layer are fully formed), weight stability is reached, and apparently all growth of soft tissues is completed. It would appear from Figure 2B that water absorption is not completed for at least two weeks following molting. This is perhaps a false impression one might obtain at first observation. Although the water content is above the late Stage C value, it must be remembered that growing tissue is gradually replacing water absorbed at and following molt. Water absorption in *Panulirus* is probably completed the day following molt. This is in agreement with the situation observed in *Maia squinado, Carcinides maenas,* and *Cancer pagurus* (Drach, 1939) in which water absorption apparently is completed within 6 to 12 hours or less following molt.

Mechanisms Involved in Swelling and Lateral Expansion of the Thorax Immediately Preceding Ecdysis

Since the animals increase their body weight approximately 10% before molting and since this increase is entirely accounted for by uptake of water, the question

arises as to how this water enters the organism. Two possible structures which might be involved in water retention were studied with the idea of obtaining evidence of the cause of the greatly expanded and swollen thorax, which results ultimately in the lifting of the exuviae at ecdysis. The major structures considered were the membranous layer of the old skeleton and the stomach.

The membranous layer

The homogenous non-laminated layer which is attached to the inside of the exuviae, observed by Vitzou (1882), Herrick (1895), Yonge (1932), and called the membranous layer by Drach (1939), was investigated as a possible site of water retention. It might be added that this layer holds large amounts of water after the exoskeleton is shed and appears to swell because of water absorption from the medium. Weight changes were followed in two premolt animals. From one animal (A) the membranous layer was removed at one day preceding molt, at which time no marked change in body weight had occurred, while in the other (B) the membranous layer was removed when the animal had increased its body weight 8% preceding molt. Wet and dry weights of pieces of this layer (as close to 2 cm^2. in size as possible) were obtained and per cent water in each piece compared. A difference in water content of the two pieces might indicate whether this structure is involved in water retention. It was found, however, that the water content in (A) was not appreciably different from that of (B). Therefore the idea that water retention by the membranous layer may contribute to weight changes preceding molt was abandoned.

The stomach

The stomach was then considered as a site for water uptake and retention preceding molt. Drach (1939) demonstrated in crabs that absorption of water during the course of shedding and shortly following occurs primarily through the gut. The stomach becomes turgid and greatly swollen during this period and in *Panulirus* swells to the extent that it occupies over half of the cephalothorax. This would cause pressure to be exerted on the surrounding regions and is, in all probability, the actual cause of swelling and lateral expansion of the thorax. Drach (1939) attributed the turgidity and swelling of the stomach in *Maia squinado* and *Cancer pagurus* to the water held in the stomach and digestive tube during shedding. He was able to remove a considerable quantity of water from the stomach of these animals, shortly preceding ecdysis, and within a short interval removal of a large quantity was again possible. In an attempt to withdraw water from the stomach of *Panulirus,* 10 premolting animals were selected. As the animals increased their weights preceding ecdysis, attempted withdrawal of water from the stomach, by inserting a cannula into the stomach via the esophagus, proved fruitless. Even when the increase in body weight had reached 8% a few minutes preceding ecdysis, little water could be withdrawn. No more than 0.5 cc. could ever be withdrawn. In three cases, animals were weighed until the body weight increased by 8–10% and then sacrificed. The old and new skeleton overlying the stomach were removed. A syringe needle was inserted in the turgid stomach in an attempt to withdraw fluid. No fluid was present. On the contrary, it was

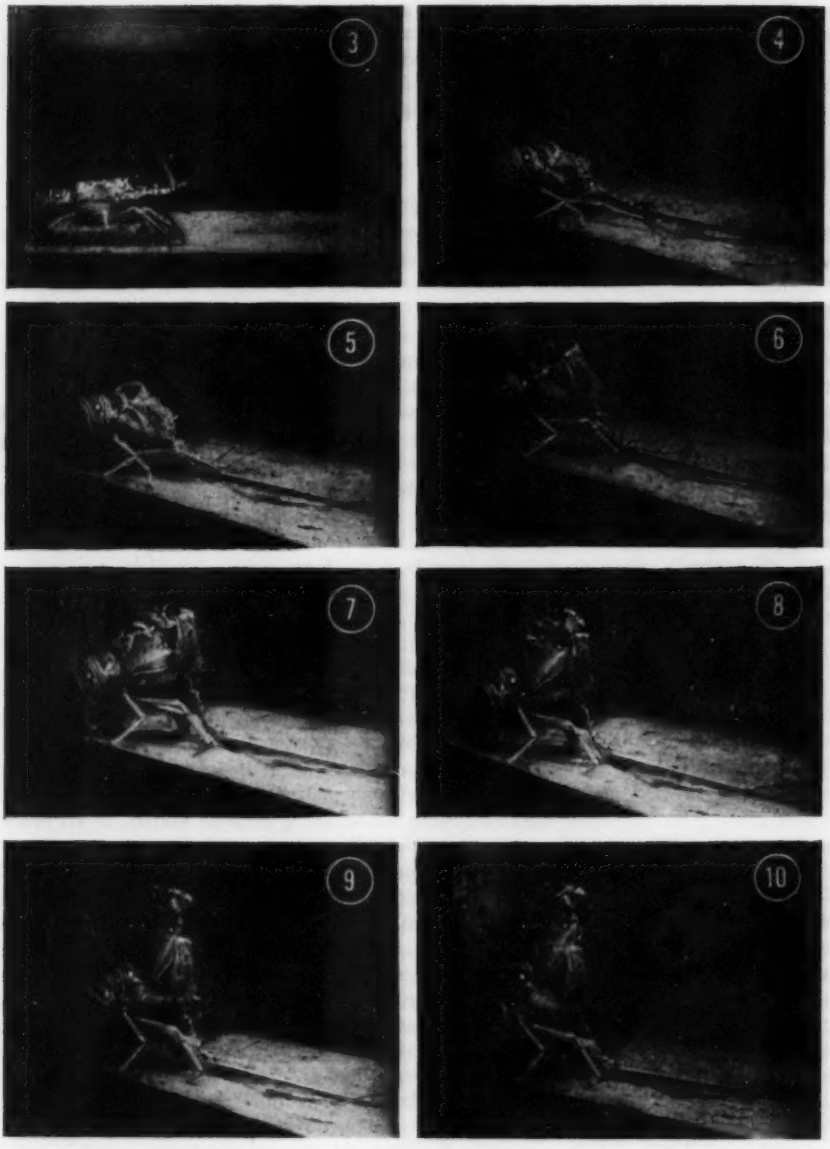

FIGURE 3. Ecdysis in *Panulirus*. The animal seeks out a suitable place to shed.
FIGURE 4. The tail is flexed; the thorax is lowered on the substratum with antennae and antennules lowered and extended to facilitate forward bracing of the animal, while the last pair

found that the turgidity and swollen condition was due to the presence of gas of unknown composition which appeared to be between the old stomach lining and the new. Following the puncture of the new outer layer with a syringe needle the wound closed and the stomach again swelled. An incision in this layer caused the stomach to collapse. The "balloon" condition of the new stomach causes the new soft body to expand in the thoracic region and is without doubt the immediate mechanism which lifts the old carapace at ecdysis. Such a mechanism would also account for the increase in volume of the emerging animal.

Water absorbed preceding ecdysis is probably taken in primarily through the gills and the soft intersegmental membranes. It does not appear to be taken in through the digestive tract. Hydration of the tissues probably occurs during the late pre-ecdysial as well as post-ecdysial period. This hydration of the tissues has been indicated by Baumberger and Olmstead (1928) in *Pachygrapsus crassipes* and is suggested by the recent work of Kincaid and Scheer (1952) on *Hemigrapsus nudus*. It is pertinent to add that the former workers showed that there was a rise in the internal osmotic pressure preceding molt. This peak in osmotic pressure is presumably caused by an increase in osmotically active constituents (organic acids) which accumulate in the hemolymph as a result of the breakdown of fats and glycogen, abundant in the hepatopancreas during the premolting period of all Crustacea investigated. The increase in osmotic pressure preceding molt would therefore account for increased water absorption preceding and immediately following molt. Increased water uptake causes the osmotic pressure to fall sharply following molt and to return gradually to normal as the skeleton of the animal hardens. A study of the green glands during the pre-ecdysial period should be made. It is quite possible that antidiuresis might occur during the few hours preceding ecdysis. Such water retention could also serve as one of the correlatives contributing to the marked increase in weight of the animal during this time. Furthermore, it is

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of walking legs is extended behind and serves as the hind bracing elements. The old carapace has begun to lift, making visible the new skeleton below the old branchiostegites.

FIGURE 5. A further stage during ecdysis showing the progressive lifting of the old carapace.

FIGURE 6. At this stage the animal has raised the thorax off of the substratum and the old carapace has lifted about 45° from its original position.

FIGURE 7. At this stage of ecdysis the animal has raised itself considerably from the substratum with the tail gently flexed. The old carapace has lifted and has tilted at an angle of 90° from its original position. The thorax of the animal is almost completely freed, making visible the new eyes and rostral spines. During this stage of ecdysis, the old branchial chamber becomes evident ventral to the new branchiostegites. It is interesting to note that the abdomen begins to undergo contractions in a telescopic fashion, *i.e.*, segments begin to telescope and extend. Freeing of the abdomen occurs in this manner.

FIGURE 8. A further stage in ecdysis. The animal has raised itself considerably from the substratum by arching its soft cephalothorax. The old branchial chamber is almost completely freed. The new antennae are just beginning to appear and two new abdominal segments are free.

FIGURE 9. In this stage of ecdysis, the animal has arched its soft cephalothorax almost 90° from its original position, three new abdominal segments are freed, the fourth beginning to appear and the new antennae are much more evident. The new 5th walking leg may be observed between the old gill chamber, old branchiostegites and the new.

FIGURE 10. The last stage of ecdysis, a second or so before the animal gives one sudden flexion and extension of the abdomen freeing itself completely from the old skeleton. It will be noted that four new segments are visible, the new proximal portions of the antennae and the new fifth walking leg are clearly evident.

possible that changes in salt excretion by the green glands might also be a factor in raising the osmotic pressure prior to molting.

Ecdysis

All of the changes observed in the premolt period lead to shedding of the exoskeleton. This is depicted in Figures 3–10. For a period of at least two hours preceding ecdysis, the spiny lobster undergoes exercise movements. These include flexing of the abdomen under the thorax and bumping it along the substratum; lowering of the thorax on the substratum while raising and extending the abdomen rigidly; lunging movements with sudden stops. These are all movements to facilitate loosening of the old skeleton from the new. Following such movements, the animal seeks a suitable place to shed (Fig. 3). The tail is then flexed; the thorax is lowered on the substratum with antennae and antennules lowered and extended to facilitate forward bracing of the animal, while the last pair of walking legs is extended behind and serves as the hind bracing elements (Fig. 4). In this position, little movement is observed other than slow contractions of the soft body beneath the old carapace. As these slow contractions occur the old carapace begins to lift at the intersegmental membrane between carapace and abdomen, making visible the new skeleton below the old branchiostegites (Figs. 4, 5). It should be pointed out again that the resorptive or ecdysial line serves as a hinge. The resorption from, and loosening of, an articulating condyle, which attaches the branchial chamber to the inner posterior edge of the branchiostegites, allow the soft body to expand in width (Figs. 5, 10). There is no breaking along the resorptive line of *Panulirus argus* as Crawford and De Smidt (1922) and Smith and Gatham (1948) thought. As the eyes are withdrawn from their old skeleton, the carapace lifts considerably (Fig. 7). At this time (Figs. 7, 10) the segments of the abdomen begin to move in and out in a telescopic fashion, thus freeing themselves from the old skeleton. As the animal pulls itself free from the old carapace it raises itself from the substratum by arching its soft cephalothorax (Figs. 8, 10). The rostral spines and eyes appear, while the old gill chamber can be seen ventral to the new branchiostegites (Figs. 7, 10). The antennae are then pulled free of the old skeleton (Figs. 8, 10). Newly withdrawn legs may be observed between the gill chamber and branchiostegites of the old skeleton (Figs. 9, 10). Three abdominal segments are observed free of the old (Fig. 9). A later stage (Fig. 10) indicates how the animal frees itself from the old skeleton. Here four abdominal segments are freed and the legs are more clearly observed above the old gill chamber between the new and old branchiostegites. The animal raises itself much above the old skeleton while pulling itself free. At this stage the animal suddenly flexes and then extends the abdomen, freeing it completely from the old skeleton. The actual time required for the entire process of ecdysis averages from 3–5 minutes for the 70–79 mm. animals depicted. The larger animals, 80–89 mm., require from 5–10 minutes. Immediately following ecdysis the animal moves about vigorously. Expansion of the flattened appendages is accomplished in this manner. By such movements, blood rushes into the appendages and causes them to assume their normal shape.

Following molting, these animals were allowed to remain isolated from all others through Stage A and B (a period of 5–6 days) at which time they begin to feed, their skeleton attains a parchment-like consistency, and they are capable of

protecting themselves. By 7–12 days (Stage C) the branchiostegites have hardened and complete hardening of the skeleton by deposition of minerals therein occurs within approximately four weeks.

Factors Influencing Molting

Temperature

In Bermuda where average monthly water temperatures range from approximately 17° in February to 29° C. in August, the range would appear to be sufficiently great to stimulate *Panulirus argus* to molt seasonally. Very little molting occurs in the months of December–April, during which time the water temperatures are lowest (Fig. 11 and Table II). It might be added that the temperature of tank water is within a few tenths of a degree of that recorded at the nearby tide station. A water temperature of 22° C. or slightly over in the spring is sufficient to initiate molting (Fig. 11). As water temperatures approach 23° C. the number of molting individuals rises from below 10% to well above. This rise begins in May and increases throughout the summer months with the peak of molts occurring in June, July, August and September, at which time the highest water temperatures of 28°–30° C. are reached. The number of molting individuals, during

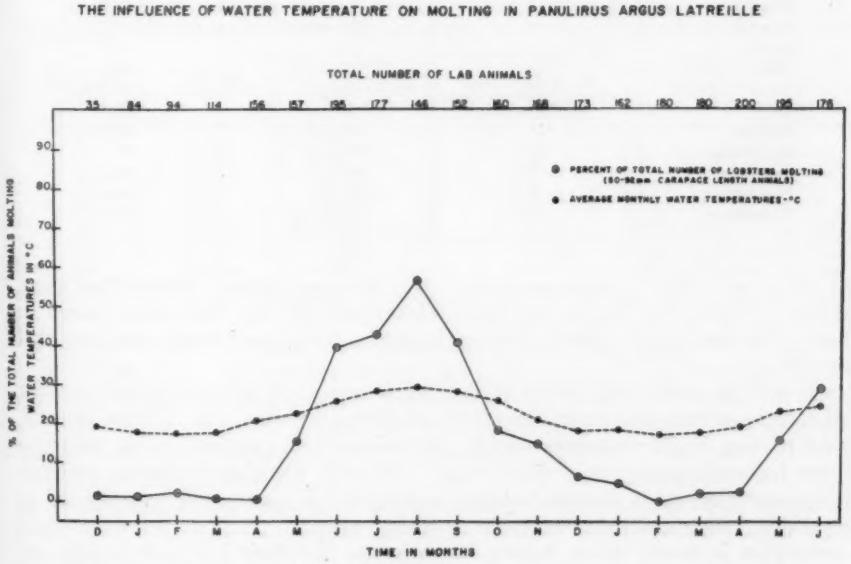
THE INFLUENCE OF WATER TEMPERATURE ON MOLTING IN PANULIRUS ARGUS LATREILLE
TOTAL NUMBER OF LAB ANIMALS
35 84 94 114 156 157 195 177 146 152 160 168 173 152 150 180 200 195 176
PERCENT OF TOTAL NUMBER OF LOBSTERS MOLTING (80-92mm CARAPACE LENGTH ANIMALS)
AVERAGE MONTHLY WATER TEMPERATURES-°C
% OF THE TOTAL NUMBER OF ANIMALS MOLTING
WATER TEMPERATURES IN °C
90
80
70
60
50
40
30
20
10
0
D J F M A M J J A S O N D J F M A M J
TIME IN MONTHS

Figure 11. The influence of water temperature on molting in *Panulirus argus*. The total number of animals in the laboratory population in any given month is indicated along the top ordinate while the respective months are indicated along the bottom ordinate. Along the Y-ordinate water temperature in ° C. and per cent of the total number of animals molting are indicated. The per cent of molting individuals is plotted with mean temperatures against time in months.

late fall, begins to decline as temperatures decrease, and in the month of December (temperature range 18°–20°) less than 10% of the total laboratory population molts. Water temperatures of 17°–18° C. virtually stop the process. The influence of the water temperatures on molting has been pointed out by Templeman (1936a, 1936b, 1940), Drach (1939, 1949), and Truitt (1939). The quantity of food required by the animals also depends largely upon the temperature. When the water temperature is low the food requirement of the animals is low. Their metabolism is greatly accelerated with a rise in water temperature. Other factors, such as photoperiodism, lunar phases, tides, and salinity of the water which might possibly influence molting, have not been investigated by the author. Templeman (1936b), however, in addition to showing that at high water temperatures larval American lobsters molted more frequently than in colder waters, found that salinities as low as 21 parts per mille were only slightly less favorable than the normal

TABLE II

Intermolt periods of 80–89 mm. carapace length animals during a period of one year

Month of molt	Number of animals molting	Intermolt periods in days	Average monthly Temperature, °C.
January	3	161±1	17.9
February	0	No molts	17.4
March	0	No molts	17.8
April	1	73	20.7
May	10	67±2	22.8
June	17	68±2	25.9
July	13	60±4	28.4
August	11	142±8	29.2
September	10	201±6	28.1
October	5	232±5	26.0
November	5	213±2	21.0
December	0	No molts	18.2

31 parts per mille. Larvae reared in total darkness molted earlier than those reared in light, were larger, and had a higher survival rate than those reared in light. He also found (1940) that egg-laying in American lobsters was retarded by cold.

It will be noted from Table II that the intermolt or growth periods are the shortest in animals molting in the months of May, June, and July. These animals, after molting in the respective months, are subjected to gradually rising temperatures and molt again within 60–70 days. Although the highest average monthly temperature occurs in August, animals molting in this month will be subjected to gradually falling temperatures in the following months. As a consequence, their metabolism is slowed down, feeding becomes less, and their intermolt periods are prolonged. This is likewise true of animals molting in September, October, November, and January. The growing period of the Bermuda lobster therefore appears to be limited effectively to a period of approximately seven months in the laboratory (the months during which the water temperatures are highest). Very little molting and growth occur in the population during the months of December

through April (the months during which the water temperatures are lowest). Sutcliffe (personal communication) has indicated that the two foregoing statements are also, in general, applicable to the field population.

Although the summer intermolt periods (Table III) are shorter in smaller than in larger animals, the actual number of molts observed during a period of one year in four of the size groups maintained in the laboratory proved to be approximately the same. Generally four molts per year were observed in these groups. This again appears to be due to the fact that the intermolt periods are prolonged following the August molt. As winter months approach, the smaller as well as the larger sexually immature animals cease to molt and grow.

The influence of size on molting

From the foregoing, water temperature does appear to be largely responsible in *Panulirus argus,* for: (1) The onset of molting; and (2) the frequency of molting and therefore the rate of growth. It is apparent that the rate of growth varies from one size group to the next. The smaller the individuals, the more frequently they molt or the more rapid are their growth rates. This was determined by

TABLE III

Average summer intermolt periods of various size lobsters molting in May, June and July

Number of animals	Carapace length	Average summer intermolt period ± Standard Error of the Mean	Theoretical number of molts per year in the laboratory based on yearly intermolt periods and growing time	Actual number of molts observed in animals maintained for one year
41	80–89	65±1	3–4	3–4
42	70–79	56±1	3–4	3–5
25	60–69	55±2	3–4	3–4
5	50–59	51±2	4–5	4
4	40–49	41±1	5	Not carried for one year
11	30–39	44±3	5	Not carried for one year
29	20–29	40±2	5	Not carried for one year

noting the summer intermolt periods of each size group maintained in the laboratory (Table III). It is evident from Table III that as animals increase in size their intermolt periods are longer or the frequency of shedding decreases. In small animals, the rate of growth is rapid and it decreases progressively as they approach sexual maturity. Kinoshita (1934), Nakamura (1940), Templeman (1940), Challenger (1943), Pearson and Anderson (1946), and Bradstock (1950) have also noted that young lobsters of several species (*Panulirus japonicus, Homarus americanus, Panulirus interruptis,* and *Jasus lalandi*) molt more frequently than old ones.

GROWTH IN LABORATORY-MAINTAINED ANIMALS

Before discussing growth of laboratory-maintained spiny lobsters, it should be pointed out that this information cannot represent exactly the growth of animals

under natural conditions, for laboratory conditions, at best, do not truly duplicate the field situation.

The following information was obtained to serve as a basis for future studies concerning hormonal factors which influence molting and growth in the Bermuda spiny lobster.

Growth by weight

Weight stability is reached several weeks following a molt. The length of this period depends upon size of the animals and the season of the year. No attempt was made in these sexually immature animals to separate the data on the basis of sex. Growth in weight is one of the most important means of studying many aspects of the molting cycle, such as determining the per cent water in the animals, the amount of water absorbed, and per cent weight change throughout the molting cycle (discussed in a previous section of this paper.) Special care, however, must be taken in handling Crustacea while following individuals through their cycle, because the antennae and periopods may be autotomized. If such happens, these animals can no longer be used for subsequent weight changes. Autotomy can generally be avoided by draining the large concrete tanks and by carefully lifting each individual out of the drained tanks for weighings.

TABLE IV

Per cent growth by weight per molt during the summer months

Number of animals	Carapace length (mm.)	Mean per cent growth by weight per molt ±standard error of the mean	Calculated per cent growth per year based on number of molts per year (Tables III, VI)	Weight stability is reached in the summer days post molt
48	80–89	5.8±0.6	17–23	28–35
33	70–79	8.4±1.0	25–34	28
22	60–69	10.0±1.1	30–40	28
20	50–59	14.9±1.7	60–75	21–28

The weight changes of the 80–89 mm. carapace length animals were observed throughout the molting cycle (Fig. 2A). These weight changes are expressed as per cents of the previous intermolt weights. The per cent growth by weight of these larger animals, throughout the summer months, indicates that they increase their body weight on the average approximately 6% per molt in the laboratory. From Table IV it will be observed that the smaller the animals, the greater the per cent growth by weight. Templeman (1940) has previously shown that the absolute increase in weight per molt of the American lobster is greater with an increase in size. An American lobster 6″ weighing 3.5 oz. gains 2 oz. per molt, (5.7%), while a 14.5″ lobster weighing 55 oz. gains 22 oz. per molt, (4.0%). Weight stability in *Panulirus* is reached earlier in smaller animals than in large ones. The period at which the skeleton is fully hardened and at which time growth of the tissues is virtually completed (Late Stage C of Drach, 1939) must be utilized in determining the per cent growth by weight. Earlier stages in the molting cycle, A, B, or early C, will give fluctuating results simply because there is a rather steady increase in weight up to Late Stage C (28 days) in *Panulirus*.

Growth by length

Growth in length is determined by the growth of the epidermis, which retracts from the old skeleton and increases its cell number or size or both about ten to fourteen days before shedding occurs in the summer months. It is at this time of retraction that the epidermis grows before the new soft skeleton begins to be deposited. Increase in length may be noted within an hour following ecdysis. By this time, the new soft skeleton has expanded to the point at which *Panulirus* reaches a size which it will retain until the following molt.

The per cent growth in length per molt for the various size classes is listed in Table V—a. It might be pointed out at this time that Tables V—a and V—b represent two different groups of animals.

It will be noted that there is a greater per cent growth by length per molt in smaller than in larger animals. This has also been observed in *Homarus americanus* by Templeman (1936a, 1940), Mackay (1926, 1929); in *Cancer magister* by Mackay and Weymouth (1935); in *Panulirus japonicus* by Kinoshita (1934)

TABLE V

V—a Per cent Growth by Length per Molt				*V—b* Gain in Carapace Length per Molt	
Carapace length (mm.)	Number of animals	Mean per cent growth by length per molt ±Standard Error of the Mean	Calculated per cent growth per year based on number of molts per year (Tables III, VI)	Number of animals	Average (mm.) gain in carapace length per molt ±Standard Error of the Mean
80–89	72	2.5±0.5	8–10	106	2.6±0.2
70–79	73	3.2±0.3	10–16	131	2.8±0.1
60–69	53	4.4±0.4	13–18	102	3.1±0.2
50–59	21	5.8±0.7	23–29	45	3.2±0.2
40–49	15	8.9±0.8	45	27	3.6±0.5

and Nakamura (1940). This difference observed in the laboratory does not appear to be due to significant differences in the gain of carapace length per molt from one size group to the next, but appears to be due to the fact that a smaller animal increasing its carapace length the same amount as a larger one (Table V—b) shows a greater per cent growth by length. There is a tendency, though not a significant one, for the smaller animals (40–59 mm. class) to increase their carapace length more per molt than the larger animals (Table V—b).

A few animals of the first four size groups (50–89 mm. carapace length) were successfully maintained for one year in the laboratory (Table VI). The number of molts, total number of mm. gained, and the per cent increase in length per year were determined. The results observed here serve as a rather good cross-check on information obtained in Table V—a and V—b from other animals.

Molting without growth

That growth by length does not necessarily accompany molting, was evidenced in some laboratory-maintained animals. Molting without growth or negative growth has also been observed in *Panulirus argus* maintained in live cars or wire

enclosures, under what this author considers as extremely poor conditions, by Marshall (1945) and Dawson and Idyll (1951). Within a period of almost two years and out of a total number of nearly 500 laboratory animals of various sizes, 27 were observed to undergo no increase in length following the first laboratory molt. Five of the 27 animals decreased in length by one mm. following molt. Seventeen of the 27 lobsters were obtained from a small, overcrowded lobster pound in which food was scarce and in which, as a result of overcrowding, most of the animals had autotomized some periopods as well as antennae. Autotomy, as a result of handling, was occasionally observed in animals brought in from the field, 10 of which are included in the 27 mentioned above. That loss of appendages markedly affects the growth of the animal as a whole was demonstrated in the American lobster by Emmel (1904, 1906). He found that when regeneration occurs, the regenerating appendages are favored over increase in body length at the first molt after injury, and that regeneration tends to lengthen the intermolt period. A previous history of unfavorable environmental conditions, such as insufficient food, overcrowding and autotomy of appendages, did not result in growth at the first molt in the laboratory, although subsequent molts by these animals were accompanied by good growth.

TABLE VI

Growth in length of animals maintained for one year in the laboratory

Class	Number of animals	Number of molts per year	Carapace length gain (mm.) per year	Mean per cent increase in length ± Standard Error of the Mean
80–89	13	3–4	9.1	10.7±1.0
70–79	15	3–5	9.7	13.2±0.9
60–69	13	3–4	12.2	19.1±1.1
50–59	2	4	11.0	21.2±2.0

SUMMARY

1. In *Panulirus argus*, premolt animals may be detected by the appearance of a resorptive or ecdysial line along the branchiostegites.

2. One of the most obvious external changes in *Panulirus* a few hours before ecdysis is the swollen condition of the intersegmental membranes.

3. Five to fifteen minutes preceding ecdysis, animals of 80–89 mm. carapace length increase their body weight by approximately 10%. This weight increase begins four to six hours preceding ecdysis and is entirely due to water absorption. There is a loss of weight following ecdysis due to loss of the exuviae. Within a few hours following molt, the weight of the individual begins to rise and continues to do so until a maximum is reached at 28–35 days. The water content begins to rise the day preceding molt, reaches its highest peak (11% above the intermolt value of 71%) one day following molt, slowly declines but does not return to its intermolt value before 28–35 days. At this period the animal has fully calcified its new skeleton, and apparently all growth of soft tissues is completed. Weight stability is not attained in 80–89 mm. carapace length animals until 28–35 days following molt.

4. Due to resorption from the ecdysial line along the branchiostegites and resorption from an articulating condyle connecting the branchial chamber to the posterior edge of the branchiostegites, progressive swelling and lateral expansion of the thorax is allowed.

5. Turgidity within the new stomach causes considerable pressure to be exerted on the surrounding regions and results in the progressive swelling and lateral expansion of the thorax preceding ecdysis. This is due to a gas which appears between the old stomach lining and the new.

6. Photographs and captions depict ecdysis in *Panulirus*.

7. The molting process appears to be directly correlated with water temperature. In Bermuda average monthly water temperatures range from 17° in February to 29° C. in August. Little molting occurs in the months of December through April, during which time the water temperatures are lowest. Water temperatures of 22° C. in May are sufficient to initiate molting. The number of molting individuals in the laboratory population increases throughout the summer months with a maximum in June, July, August, and September, at which time the highest water temperatures of 28–30° C. are reached. This means that the intermolt periods are shortest during the summer months. The number of molting individuals during late fall declines, as temperatures decrease. Water temperatures of 19° C. or below, however, are required to stop the molting process in the winter. Decrease in molting frequency in the fall seems to be a consequence of decreased metabolism.

8. The growing period of the Bermuda lobster appears to be limited effectively to approximately seven months in the laboratory (May–November).

9. Smaller individuals grow more rapidly in the summer months than do larger individuals. Although the summer intermolt periods are shorter in smaller than in larger animals, the actual number of molts observed during a period of one year in four of the size groups maintained in the laboratory (50–89 mm. carapace length) proved to be approximately the same. An average of four molts per year was observed in these groups. As winter months approach, the smaller as well as the larger animals cease to molt and grow.

10. The smaller the animals, the greater the per cent growth by weight. Larger animals, however, gain more actual weight than smaller animals per molt.

11. There is a greater per cent growth by length per molt in smaller than in larger animals. The difference observed between larger and smaller animals is an apparent one because a smaller animal increasing its carapace length the same amount as a larger one shows a greater per cent growth by length.

12. Growth by length does not necessarily accompany molting. This was evidenced in some laboratory-maintained animals. In virtually all of these cases the environmental conditions of the individuals were poor.

LITERATURE CITED

BAUMBERGER, J. P., AND J. M. D. OLMSTEAD, 1928. Changes in osmotic pressure and water content of crabs during the molt cycle. *Physiol. Zool.*, **1**: 531–544.

BRADSTOCK, C. A., 1950. A study of the marine spiny crayfish *Jasus lalandii* (Milne-Edwards). *Zool. Publ. Victoria Univ. College*, **7**: 1–38.

CHALLENGER, T., 1943. The story of the crayfish. *Tasmanian J. of Agriculture*, **14**: 51–55.

CRAWFORD, D. R., AND W. J. J. DE SMIDT, 1922. The spiny lobster, *Panulirus argus*, of southern Florida: Its natural history and utilization. *Bull. of Bur. Fish.*, **38**: 281–310.

DAWSON, C. E., AND C. P. IDYLL, 1951. Investigations on the Florida spiny lobster *Panulirus argus* (Latreille). *State of Florida Board of Conservation Technical Series*, **2**: 6–39.

DRACH, P., 1939. Mue et cycle d' intermue chez les crustacés decapods. *Ann. Inst. Oceangr.*, **19**: 103–391.

DRACH, P., 1949. Périodes de mue chez un Crustacé à croissance lente (*Xantho floridus* Montagu) dans le district septentrional de son aire de reproduction. *C. R. Acad. Sci.*, **228**: 128–130.

EMMEL, V. E., 1904. The regeneration of lost parts in the lobster. Thirty-fifth annual report of the Commission of Inland Fisheries of R. I. Special Paper No. 20: 81–117.

EMMEL, V. E., 1906. The relation of regeneration to the molting process of the lobster. Thirty-sixth Annual Report of the Commission of Inland Fisheries of R. I. Special Paper No. 27: 257–313.

FISHER, R. A., 1948. Statistical methods for research workers. 10th ed. Hafner Pub. Co., Inc., New York.

GUYSELMAN, J. B., 1953. An analysis of the molting process in the fiddler crab, *Uca pugilator*. *Biol. Bull.*, **104**: 115–137.

HERRICK, F. H., 1895. The American lobster. *Bull. U. S. Fish Comm.*, **15**: 1–252.

KINCAID, F. D., AND B. T. SCHEER, 1952. Hormonal control of metabolism in crustaceans. IV. Relation of tissue composition of *Hemigrapsus nudus* to intermolt cycle and sinus gland. *Physiol. Zool.*, **25**: 372–380.

KINOSHITA, T., 1934. On the artificial propagation of *Panulirus japonicus* and some account of its larvae. *Proc. 5th Pan. Pacific Sci. Cong.*, **5**: 4191–4193.

MACKAY, D. A., 1926. Rate of growth of young lobsters. *Science*, **64**: 257.

MACKAY, D. A., 1929. Larval and postlarval lobsters. *Amer. Nat.*, **63**: 160–170.

MACKAY, D. C. G., AND F. W. WEYMOUTH, 1935. The growth of the Pacific edible crab, *Cancer magister* Dana. *J. Biol. Board Canada*, **1**: 191–212.

MARSHALL, N., 1945. The molting without growth of spiny lobsters, *Panulirus argus*, kept in a live car. *Trans. Amer. Fish. Soc.*, **75**: 267–269.

NAKAMURA, S., 1940. Ecological studies on the spiny lobster *Panulirus japonicus* (V. Siebold) with special reference to its conservation. *J. Imper. Fish. Inst.*, **1**: 101–113.

PEARSON, J. C., AND W. W. ANDERSON, 1946. Spiny lobster. *U. S. Fish and Wildlife Service Fishery Leaflet*, **142**: 1–4.

SMITH, F. G. W., AND C. A. GATHMAN, 1948. The spiny lobster and scalefish industry of British Honduras. Report from the Univ. Miami Mar. Lab. 1–29.

SUTCLIFFE, W. H., JR., 1952. Some observations of the breeding and migration of the Bermuda spiny lobster, *Panulirus argus*. Proc. Gulf Carib. Fish Insti., Fourth Annual Session: 64–69.

SUTCLIFFE, W. H., JR., 1953. Further observations on the breeding and migration of the Bermuda spiny lobster, *Panulirus argus*. *J. Mar. Research*, **12**: 173–183.

TEMPLEMAN, W., 1936a. Local differences in the life history of the lobster (*Homarus americanus*) on the coast of the maritime provinces of Canada. *J. Biol. Board Canada*, **2**: 41–87.

TEMPLEMAN, W., 1936b. The influence of temperature, salinity, light and food conditions on the survival and growth of the larvae of the lobster (*Homarus americanus*). *J. Biol. Board Canada*, **2**: 485–497.

TEMPLEMAN, W., 1940. The life history of the lobster. Newfoundland Govt. Dept. of Nat. Resources Fisheries Service Bull., No. 15: 1–42.

TRAVIS, DOROTHY F., 1951a. Calcium metabolism in the decapod Crustacea. Thesis for Ph.D. degree, Radcliffe College.

TRAVIS, DOROTHY F., 1951b. Early stages in calcification of the skeleton of *Panulirus argus* Latreille. *Anat. Rec.*, **111**: 124.

TRUITT, R. V., 1939. The blue crab. Contribution No. 27 from the Chesapeake Biological Laboratory: 10–37.

VITZOU, A. N., 1882. Recherches sur la structure et la formation des teguments chez les crustacés décapodes. *Arch. Zool. Exp. et Gén., Série I*, **10**: 451–576.

YONGE, C. M., 1932. On the nature and permeability of chitin. I. The chitin lining of the foregut of decapod Crustacea and the function of the tegumental glands. *Proc. Roy. Soc. Lond., Ser. B*, **111**: 298–329.

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